

CRANFIELD UNIVERSITY

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**EFFECT OF MILSANA CONCENTRATION IN METABOLISM OF
SOLANUM LYCOPERSICUM PLANTS GROWN UNDER SALINITY
STRESS**

CRANFIELD HEALTH

MSc THESIS

2013

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School of Health

MSc Thesis

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**EFFECT OF MILSANA CONCENTRATION IN METABOLISM OF *SOLANUM*
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2013

To my parents...

Abstract

Salinity is a major abiotic factor that causes serious problems in agriculture around the world which leads to reduced crop production. Scientists all over the world are looking for natural, environment friendly compounds against biotic and abiotic factors. Milsana, a formulated plant extract from *Reynoutria sachalinensis*, is widely used to elicit plant defences against biotic stress. In addition Milsana has been found to act by enhancing the flavonoid metabolic pathway. Considering the above the effect of Milsana concentration on tomato plants under physiological conditions and under NaCl stress was examined. The experimental approach included the study of the effect of various concentrations of Milsana (0.5%, 1.0% and 2.0%) on untreated plants and plants under salinity stress (70mM and 140mM NaCl). Plant height, fruit quality, antioxidant and photosynthetic capacity were used to determine the response of tomato plants to Milsana, NaCl stress, and their combination. The effect of Milsana was tested in two different sets of experiments, one carried out in growth chambers under controlled conditions and the other carried out in a greenhouse. Milsana led to enhanced growth, when applied to non saline stressed plants. Furthermore the negative effect of NaCl on photosynthesis, ROS homeostasis, and fruit quality traits was counteracted by the application of Milsana.

Acknowledgements

I would like to extend my sincere appreciation and thanks to my supervisors Prof. Konstantinos Loulakakis, and Dr. David Aldred for their valuable guidance throughout this project and consideration of this thesis. I am grateful to Dr. Dimitrios Lydakis for giving me the opportunity to acquire this scientific experience.

I specially thank Cranfield University and the Technological Institute of Crete for their support and of letting me use all the necessary equipment needed for this project.

Grateful thanks are given to the Assistant Professor Dragassaki for supporting and encouraging me. I would also like to thank my colleagues at the Institute, especially to Maragakis Aristidis, for their great encouragement for the completion of this project. Sincere thanks to Konsolakis Giorgos, Andronis Efthimios and Christofaki Maria for their helpful advice, patience and technical support during my experimental work.

Grateful thanks to my family and my friends (especially to Makri Eleana) for supporting and encouraging me throughout the long period of this study.

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Notation

ABTS	2,2'azinobis-(3-ethylbenzothiaziline-6sulfonate)
APX	Ascorbate Peroxidase
ATP	Adenosine 5'-TriPhosphate
BER	Blossom-end rot
Ca ²⁺	Calcium
CI	Chalcone isomerase
CS	Chalcone synthase
Dw	Dry weight
EC	Electrical conductivity
Fv/Fm	Fv/Fm: I PSII photochemical efficiency)
FW	Fresh weight
H ₂ O ₂	Hydrogen peroxide
Hg/Ha	Hectogram per Hectare
MDA	MalonDiAldehyde
Mg ²⁺	Magnesium
Milsana	a formulated plant extract from <i>Reynoutria sachalinensis</i>
NaCl	Sodium Chloride
O ₂ ^{•-}	Superoxide radical
PCD	Programmed cell death

PSII	Photosystem II
ROS	Reactive Oxygen Species
TBARS	TBA Reactive Substances
TCA	TrichloroAcetic Acid
TEAC	Trolox Equivalent Antioxidant Capacity
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TBA	ThioBarbituric Acid
v/v	volume/volume
μmol	micromoles

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CHAPTER 1

Introduction

1.1 General Introduction - Problems of Saline Irrigation Water on the Island of Crete (Greece)

Crete is suffering from an increasingly severe water shortage coupled with declining groundwater supplies (Maton et al., 2010). In the south-eastern part of Crete, the groundwater with high salt content (mainly Na^+ and Cl^- due to sea water intrusion) is commonly used for irrigation (Chartzoulakis and Klapaki, 2000).

On Crete, water salinity is a major problem for agriculture. Mean precipitations per year is low (ranges between 400 and 500 mm) and unevenly distributed. The temperature is normally high and varies from 35 to 40° C. Therefore, the need for irrigating water is extremely high. The intensive use of the underground sources of water results in increased intrusion from sea water especially in coastal areas. Also, due to the lack of sufficient quantity of acceptable quality water, in some areas water from saline springs is used. Especially in the area of Ierapetra, where more than 1,100 hectares of vegetables are grown in greenhouses, the critical factor for a successful cultivation is the water quality.

Tomatoes and cucumbers are the main vegetables grown in the area in greenhouses and both of them suffer from the continuously increasing salinity in the irrigating water. In addition to the water salinity, the intensive use of chemical fertilizers in greenhouses significantly increases the electrical conductivity (EC) and has a negative effect on the plants.

1.2 Abiotic and Biotic Factors that Affect Plants

Plants are naturally subjected to two different types of stresses, abiotic and biotic. Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment and is produced by natural factors such as extreme temperatures, wind, drought, and salinity (Maes and Debergh, 2003).

Biotic stress is caused by living organisms which can harm plants, such as viruses, fungi, and bacteria (Maes and Debergh, 2003).

Whereas a biotic stress would include such living disturbances such as fungi or harmful insects, abiotic stress factors, or stressors, are naturally occurring, often intangible, factors such as intense sunlight or wind that may cause harm to the plants and animals in the area affected. In essence all extreme values (minima or maxima) of environmental factors such as light intensity, temperature, moisture, salinity, drought and air pollutants can all be classified as abiotic stress factors (Fig. 1.1). The common aspect of all abiotic stress factors mentioned above is that they are essentially unavoidable (Vickers et al., 2009).

Therefore in order to cope and eventually survive, plants deviate from their normal operating state, leading to abnormal physiological growth, low productivity, even death. Research has also shown that abiotic stressors are most harmful when they occur together. In total, abiotic stress factors are the most harmful concerning the growth and productivity of crops worldwide and threaten plants that have not been adapted and are not able to rapidly acclimatize (Vickers et al., 2009). Drought, salinity, extreme temperatures, chemical toxicity and oxidative stress, are globally and regionally important factors that have negative effect on plant physiological development. Two of the most important factors, which are usually manifested as osmotic stress, are drought and salinity (Wang et al., 2001).

Salinity in particular is one of the most harmful abiotic factors affecting agricultural crops world-wide. Salinity reduces crop productivity, plant vigour and crop yield. Plant salt stress is caused mainly due to the use of brackish water, resulting in millions of hectares of irrigated land being damaged every year as a result of high salinity levels in the soil (Munns and Tester, 2008).

Excess salts in soil lead to ion cytotoxicity and osmotic stress (an increase of osmotic pressure in the soil due to the increase in ions by excess NaCl, which prevents water uptake by the plant) and decreased uptake of nutrients. Resulting in metabolic imbalances which lead to oxidative stress (a shift of the balance between prooxidative and antioxidative reactions in favour of the former, a common denominator of the action of various agents on living organisms) (Bartosz, 1997; Foyer and Shigeoka, 2011; Tuteja, 2007).

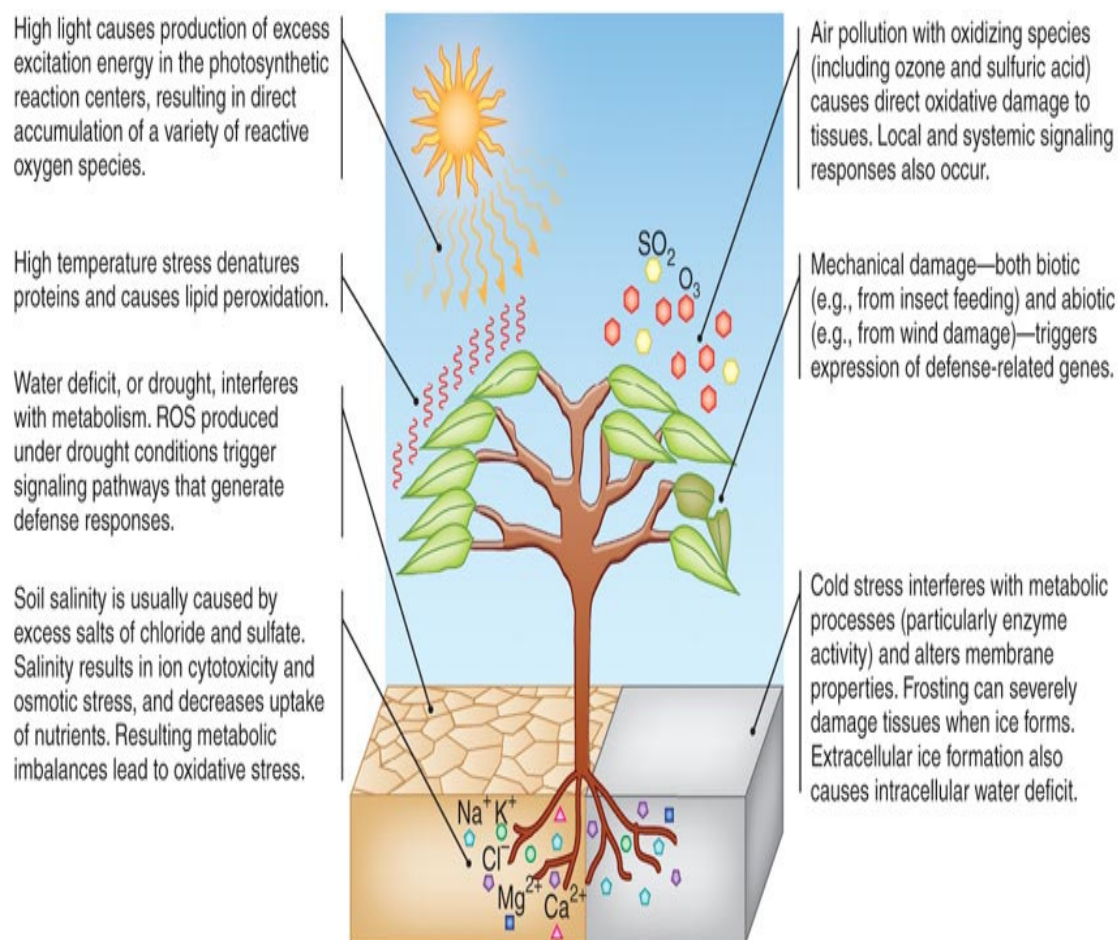


Figure 1.1: Key stress factors such as light Intensity, Temperature, Soil Salinity, Moisture and other Abiotic Factors which impact on the Plant Environment (Vickers et al., 2009)

In order to “survive” when subjected to salt stress plants adapt to the environment. Several biological and chemical pathways are activated in plants to protect their chloroplast functions and other major organelles, and maintain ion homeostasis. Salinity stress increases the production of Reactive Oxygen Species (ROS), highly reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2) and superoxide ($\text{O}_2^{\bullet-}$), in plants. Depending on their genetic capacity plants have developed enzymatic and non-enzymatic defence systems against ROS. Osmotic stresses caused by salinity promote oxidative stress and plants with high constitutive and induced antioxidant levels have better resistance to damage (Parida and Das, 2005).

1.3 Effect of Salinity Stress in Plants

The most important environmental stress, which considerably reduces crop production on at least 20% of cultivated land worldwide, is high salinity. Various genes, the products of which are involved in plant protection, are up regulated due to high salinity stress. Some genes, encoding products such as osmolytes, ion channels, receptors, components of calcium signalling, and some other regulatory signalling factors or enzymes, can be transferred to plants with high sensitivity to salinity, by means of genetic engineering, to improve plant tolerance (Tuteja, 2007). Overall, the susceptibility or tolerance to high salinity stress in plants is a coordinated action of multiple stress responsive genes and also cross talk with other components of stress-signal transduction pathways. Salinity stress could also lead to growth inhibition or cell death (Fig. 1.2), which will depend upon how many and which kind of genes are up- or down-regulated (Mahajan et al., 2008); (Tuteja, 2007).

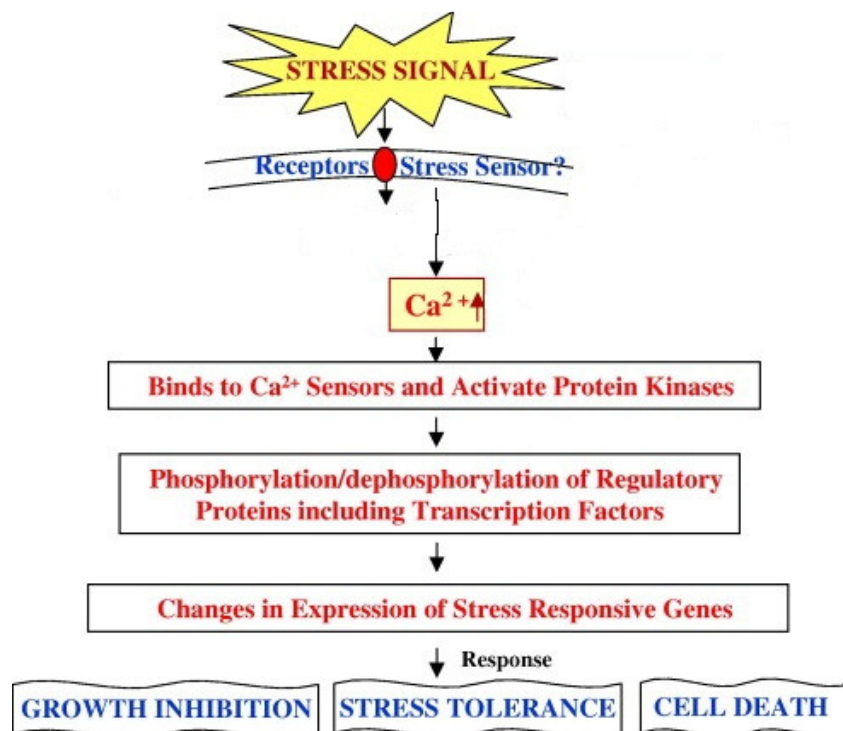


Figure 1.2: Generic pathway for plant response to stress from Mahajan et al. (2008)

In the south-eastern part of Crete the groundwater has a high salt content (particular Na^+ and Cl^- because of sea water intrusion) in commonly used for irrigation. One of the problems of this situation is that the external NaCl salinity of up to 50 mM delays seed germination (Chartzoulakis and Klapaki, 2000). In these conditions, plants activate some mechanisms precluding Na^+ and Cl^- from entering the cells and some other mechanisms that can contribute to salt tolerance of plants. Understanding these mechanisms of stress tolerance, along with a plethora of genes involved in the stress signalling network, is important to improve high salinity stress tolerance in crop plants (Tuteja, 2007).

The most serious cause of high salinity is irrigation combined with poor drainage, because it causes a decline of productivity of the agricultural land. A plant damaged by high salinity may suffer reduced shoot and root growth, yield losses and eventual death (Zhu, 2001). The most common effect of salinity on plant appearance is a general stunting of growth. Plants usually appear normal, although if compared with non-stressed plants, they may have darker green leaves that, in some cases, are thicker and more succulent. Visual symptoms, such as leaf burn, necrosis, and defoliation occur in some species, particularly woody crops, but these symptoms are rare in herbaceous crops unless plants are severely stressed. Consequently, it is difficult to diagnose a moderately salt-affected crop in the field without having a non-stressed crop nearby for comparison (Maas, 1993).

The irrigation water contains calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+). When the water evaporates, Ca^{2+} and Mg^{2+} often precipitate into carbonates, leaving Na^+ dominant in the soil. The high concentration of Na^+ in the soil causes the repression of nutrient-ion activities and produce extreme ratios of $\text{Na}^+/\text{Ca}^{2+}$ or Na^+/K^+ (Grattan and Grieve, 1998). Increases in cations and their salts, NaCl in particular, in the soil generates external osmotic potential that can prevent or reduce the influx of water into the root. The resulting water deficit manifests in a manner similar to that of drought conditions and is enhanced by the presence of Na^+ ions (Bohnert, 2007). In contrast to this general response, the yield decline after the tolerance threshold is reached is represented by a single regression line with a species-specific slope (Maas, 1990).

1.4 *Solanum lycopersicum*

Solanum lycopersicum (tomato) is a plant which belongs to the Solanaceae family. Tomato is considered as a crop of significant value worldwide. Horticultural crop production in greenhouses has evolved considerably during the latter part of the 20th century (Fig. 1.3, 1.4). About 70 million tons are produced per year representing 17% of total vegetable production (Nicot and Baille, 1996). According to F.A.O. (2007), 120 million tons of tomatoes are produced globally (annual production in Greece from 2005 to 2010 is presented in Table 1.1).

Crete in particular, because of its suitable climate, is a geographic region where large amounts, nearly 400,000 tons of vegetables are grown, especially tomatoes and cucumbers during winter and spring.



Figure 1.3: Tomato fruit and tomato plant

The importance of tomato as a vegetable and salad commodity is reflected by the large volume of research on virtually all aspects of the crop. Since 2000, over a thousand scientific papers per year relating to tomato research have been published, not including those in the less widely read literature of conference and workshop proceedings.



Figure 1.4: Tomato plants grown in greenhouses

Table 1.1: Area Harvested, Yield and Production of tomato (*Solanum lycopersicum*) in Greece from 2005 to 2010 (FAOSTAT, 2010)

Element	Year	Unit	Value	Source
Area Harvested	2005	Ha	34700	Official data
Area Harvested	2006	Ha	33881	Official data
Area Harvested	2007	Ha	33002	Official data
Area Harvested	2008	Ha	25000	Official data
Area Harvested	2009	Ha	32350	Official data
Area Harvested	2010	Ha	28000	FAO estimate
Yield	2005	Hg/Ha	493827	Calculated data
Yield	2006	Hg/Ha	463011	Calculated data
Yield	2007	Hg/Ha	443864	Calculated data
Yield	2008	Hg/Ha	535440	Calculated data
Yield	2009	Hg/Ha	482630	Calculated data
Yield	2010	Hg/Ha	502214	Calculated data
Production	2005	tonnes	1713580	Official data
Production	2006	tonnes	1568730	Official data
Production	2007	tonnes	1464840	Official data
Production	2008	tonnes	1338600	Official data
Production	2009	tonnes	1561310	Official data
Production	2010	tonnes	1406200	Official data

1.5 Effect of Salinity in Tomato Plants

Among several agricultural problems around the world, salinity appears to be the most important. Tomato plants were primarily moderately sensitive to salinity (Maas and Hoffman, 1977), but increase their tolerance to high salinity (50 mm NaCl) by adjusting their root structure (Rodriguez et al., 1997). The fruits of saline-treated plants are smaller and they have higher soluble solids than that of the fruits of plants irrigated with non-saline water (Mizrahi, 1982). In tomato plants grown under salinity stress blossom-end rot (BER) is often observed, therefore the fruits produced are unsuitable for sale (unmarketable) (Cuartero and Fernández-Muñoz, 1998).

Salinity is a complex environmental constraint that incorporates two main aspects, an osmotic one due to an increase in the external osmotic pressure of the soil solution and an ionic one linked to the accumulation of ions which become toxic at high concentrations (primarily Na^+ and Cl^-). The combination of these aspects leads to a decrease in the absorption availability of essential elements (such as calcium) (El-Bassiouny et al., 2004). Blossom-end rot (BER) is a physiological disorder that occurs wherever tomatoes (and peppers) are grown (Fig. 1.5).



Figure 1.5: Blossom end rot on NaCl treated tomato fruits

It is a non-infectious disease caused by low levels of calcium in tomato fruit. BER occurs frequently where large fluctuations in soil moisture are allowed to occur. Rapid plant growth, low potassium and calcium, excess magnesium and nitrogen, excessive salinity, root damage, and high relative humidity all predispose plants to BER. Cultivars

differ in their susceptibility to BER. Increasing salinity leads to higher occurrence of BER in tomato fruits and is also known to have negative effects in water uptake and plant growth (Albahou and Green, 1997).

The deficiency of Ca^{2+} has a negative effect on growth of tomato fruits. One of the consequences of this deficiency/problem is the susceptibility of tomato fruits to blossom end rot. Increase of salinity results in a linear decrease of the rate of Ca^{2+} uptake (Adams and Ho, 1993).

1.6 Changes in Proline Accumulation, Antioxidant System and Lipid Peroxidation in Relation to the Tolerance to Salt Stress in Tomato Plants

Proline is an amino acid, which acts as an organic solute and plays a major role in this osmotic adjustment of salt stressed plants. Biochemical analysis of tomato leaves and mature fruits for proline accumulation has shown that proline accumulation increases with increase in salt stress (Babu et al., 2012).

Under non-saline conditions, cultivated tomatoes show high induction of all antioxidants except peroxidase. When they are exposed to salt stress conditions, under 150 mM NaCl, soil salinity leads to an increase in the antioxidant content and activity. Tomato plants acclimated to salinity stress exhibit a complex antioxidant response. There are loci that control the antioxidant content under all conditions while others are responsible for antioxidant content, only under saline or non-saline conditions (Frary et al., 2010).

It has been reported that irrigation of cultivated tomatoes with seawater may result in enhanced fruit antioxidant activity, a manifestation of salt induced oxidative stress. Antioxidant compounds (also called nonenzymatic antioxidants) such as phenolic compounds, ascorbic acid, tocopherols, glutathione and carotenoids are often “recruited” by plants to quench ROS. Phenolics are water-soluble antioxidants which readily neutralize ROS by donating their hydrogen atoms. They are of particular

importance due to their prevalence in plants and the significant contribution they make to water-soluble antioxidant activity. There are more than 8,000 known phenolic compounds with flavonoids being the most common group of polyphenols in plants. Although lipid-soluble antioxidants like carotenoids are also important scavengers of ROS, their relative contribution to total antioxidant activity in fruits and vegetables is much lower than the contribution from water-soluble antioxidants (Frary et al., 2010).

High levels of H₂O₂ result in lipid peroxidation and membrane damage. Salt treatment has been demonstrated to increase lipid peroxidation or induce oxidative stress in plant tissues. Lipid peroxidation refers to the oxidative degradation of lipids. Research has reported that salt tolerance differs according to the duration of salt stress and in particular in the case of 500 mM NaCl, 12 days seem to have an intermediate effect on lipid peroxidation and antioxidative enzyme activities (Bor et al., 2003).

1.7 Milsana®- a Formulated Plant Extract from *Reynoutria sachalinensis*

Scientists all over the world are looking for natural, environment friendly prophylactic compounds that will be harmless to beneficial insects and animals.

Some of these formulations identified in the literature are:

- Chitoplant (a formulation of chitosan) which reduced powdery mildew on greenhouse tomatoes (Whipps and McQuilken, 2009)
- Sporodex (a formulation of *Pseudozyma flocculosa*) is a biological fungicide containing 1.3 % *Pseudozyma flocculosa* intended for the control of powdery mildew (Konstantinidou-Doltsinis et al., 2007)
- Messenger (a naturally occurring protein derived from *Erwinia amylovora*) it possesses efficacy against blue mould in apples and *Phytophthora infestans* on tomatoes (Fontanilla et al., 2005; Walters and Fountaine, 2009).

Milsana, a formulated plant extract from *Reynoutria sachalinensis*, is widely used to elicit plant defences against plant pathogens (Fofana et al., 2002; Randoux et al., 2006).

Randoux et al., (2006) reported that application of the extract to wheat plants led to a reduction in levels of lipid peroxidation.

Reynoutria sachalinensis (F. Schmidt ex Maxim.) Nakai is a plant (Fig. 1.6) which belongs to the Polygonaceae family and native to north-eastern Asia, in northern Japan and Russia. It is used as a medicinal plant in China, Korea and Japan. Particularly the root of genus *Reynoutria* has been used as a traditional Chinese medicine for treatment of arthralgia, jaundice caused by damp-heat, amenorrhea, cough with expectoration, scalds and burns, traumatic injuries, carbuncles and sores. Early studies have reported that a large number of compounds were isolated from the genus *Reynoutria*, including anthraquinones, stilbenes, flavonoids and other phenolic compounds. The biological activity and chemical constituents of *R. sachalinensis* have not yet been reported (Zhang et al., 2005).



Figure 1.6: Plants of *Reynoutria sachalinensis*

From the wide variety of “ingredients” found in Milsana, flavonoids are of particular interest since they have been widely recognized as photoprotective and antioxidative compounds, and are known to accumulate in plants under stress (Hernández and Van Breusegem, 2010). Flavonoids are also known to participate in the management of light

energy and carbon when the plant is subjected to growth limiting conditions (e.g. drought, low temperatures).

Furthermore, disease assessments and cytological investigations provided valuable information on the modes of action and efficacies of Milsana, against powdery mildew development on long English cucumber. Milsana application significantly reduced disease incidence relative to inoculated controls through induction of localized resistance. Microscopic observations on powdery mildew on cucumber revealed a pathogen decrease in the localized Milsana treatment and suggest that phenolics are possibly involved in the Milsana defense response (Wurms et al., 1999).

In addition Milsana has been found to act by enhancing the flavonoid metabolic pathway. Treatment of cucumber plants resulted to an increase in the levels of two genes responsible for the expression of two enzymes of the flavonoid biosynthetic pathway, namely chalcone synthase (CS) and chalcone isomerase (CI) (Fofana et al., 2002). CS is responsible for the synthesis of chalcone, a flavonoid precursor, whereas CI is responsible for the conversion of chalcone to members of the flavonoid group. Under stress conditions, plant cells have the ability to prevent water loss and to maintain continuous growth. Of particular interest are stresses where flavonoids have been known to contribute, such as salinity (Walia et al., 2005) and heavy metals (Keilig and Ludwig-Müller, 2009).

In the past, Milsana has been found to improve resistance to the powdery mildew of tomato (Konstantinidou-Doltsinis et al., 2006). Milsana was also extremely effective against powdery mildew of cucumbers, especially in greenhouse crops grown under high disease pressure. Efficacy reached 90% and was comparable to chemical fungicides (Konstantinidou-Doltsinis and Schmit, 1998). As described above the proposed model for conferring biotic stress resistance in tomato and cucumber involves members of the flavonoids family which are widely recognised for their protective role against abiotic stress on plants and particularly salinity stress (Walia et al., 2005). Therefore there is a great potential for a protective role of Milsana against abiotic stress.

1.8 Aims and Objectives

Considering the above we proposed to evaluate the effect of the Milsana, a plant extract of *R. sachalinensis*, on the physiology and growth of tomato plants. Furthermore we determined the ability of Milsana to facilitate plant resistance to abiotic stresses, such as salinity (NaCl). Tomato (*Solanum lycopersicum*) is a good candidate to test the ability of Milsana to confer abiotic stress resistance, because of the beneficial action of Milsana towards resistance to powdery mildew in tomato, and its commercial value.

The experimental approach included the study of the effect of various concentrations of Milsana on non-saline plants and plants under salinity stress (NaCl). A combination of treatments was made on intact plants grown in growth chambers and greenhouse. A set of parameters such as plant height, fruit quality, antioxidant and photosynthetic capacity will be used to determine the response of tomato plants to Milsana, NaCl stress, and their combination.

- Growth Chamber Experiments: Tomato (*Solanum lycopersicum*) seedlings (15cm in height) were grown in a growth chamber for a period of 9 weeks. Conditions in the growth chamber were $22\pm1^{\circ}\text{C}$ with a 16h light / 8h dark photoperiod. At the end of the experiment, leaf tissue samples were harvested and used for further analysis.
- Greenhouse Experiments: Tomato plants were grown in a greenhouse for a period of 13 weeks. When plants were approximately 70 cm in height, treatments were carried out, until the plants produced fruits. At the end of the experiment, leaf tissue samples were harvested, from the upper (the youngest fully expanded leaf near the tip) and lower (3rd-4th leaves from the base) zone of the plants and used for further analysis.

CHAPTER 2

Materials and Methods

2.1 Experimental Design

One of the experiments (Growth Chamber Experiment) presented in this work was established in a growth chamber and the other (Greenhouse Experiment) in a greenhouse at the farm of the Technological Education Institute (TEI) of Crete. They were arranged in a completely randomized design with 10 replicates. At the end of the experiments tissue samples were harvested and further analyzed at the Plant Physiology and Biotechnology laboratory of the School of Agricultural Technology of T.E.I of Crete.

2.2 Growth Chamber Experiment

Tomato seedlings (Fig. 2.1), were commercially obtained and transferred to pots approximately 2L in volume. Each pot was filled uniformly with 2 L homogenous Potgrond P (Klasmann-Deeilmann, German) substrate that contained a blend of black sphagnum peat. The seedlings were grown in a growth chamber (Fig. 2.2) for a period of 2 weeks prior to treatments, during which they were irrigated once a week. Conditions in the growth chamber were $22 \pm 1^\circ\text{C}$ and 16h light / 8h dark photoperiod.



Figure 2.1: Tomato seedlings (*Solanum lycopersicum*) before they were transferred to pots

At the end of this period, when plants were approximately 15 cm in height, treatments were carried out using:

- a) different concentrations of Milsana (0.0, 0.5, 1.0, and 2.0 % v/v)
- b) different levels of NaCl (0 mM, 70 mM and 140 mM) and
- c) their combinations (Table 2.1, 2.2)

Plants irrigated and sprayed with distilled H_2O , were used as controls. Treated plants were irrigated once weekly with a solution of NaCl and Milsana was applied to relevant plants, by spraying on the leaves, once a week for a period of 9 weeks (63 days), (Tables 2.1, 2.2). At the end of the experiment, leaf tissue samples were harvested and

used for further analysis. Time points and intensity of treatments were selected according to preliminary experiments.



Figure 2.2: Tomato seedlings (*Solanum lycopersicum*) grown in a growth chamber

Table 2.1: Concentrations of Milsana in the different treatments

TREATMENTS	Concentrations of Milsana, % v/v
Control	0.0
Milsana 1 (M 1)	0.5
Milsana 2 (M 2)	1.0
Milsana 3 (M 3)	2.0

Table 2.2: Concentrations of NaCl and Milsana in the different treatments

TREATMENTS	CONTENTS
Control	H ₂ O
Sodium Chloride (NaCl1)	70 mM NaCl
NaCl 1 + Milsana 1 (NaCl1-M1)	70 mM NaCl + 0.5% v/v Milsana
NaCl 1 + Milsana 2 (NaCl1-M2)	70 mM NaCl + 1.0% v/v Milsana
NaCl 1 + Milsana 3 (NaCl1-M3)	70 mM NaCl + 2.0% v/v Milsana
Sodium Chloride (NaCl2)	140 mM NaCl
NaCl 2 + Milsana 1 (NaCl2-M1)	140 mM NaCl + 0.5% v/v Milsana
NaCl 2 + Milsana 2 (NaCl2-M2)	140 mM NaCl + 1.0% v/v Milsana
NaCl2 + Milsana 3 (NaCl2-M3)	140 mM NaCl + 2.0% v/v Milsana

2.2.1 Morphological Characteristics and Growth Parameters

The morphological characteristics of tomato plants were monitored for the duration of the experimental period and the symptoms were observed. Total height of plants was measured to provide an estimate of growth for the treated and control plants. Measurements were obtained once a week for the duration of the experiment.

2.2.2 Chlorophyll Content

Chlorophyll plays an important role in the trapping of solar energy for plant photosynthesis and the measurement of chlorophyll content is an important aspect for determine photosynthetic characteristics. The *in vivo* total chlorophyll content was estimated as SPAD (Soil Plant Analysis Development) values using a SPAD-502 apparatus (Minolta Co. Ltd., Japan) (Fig. 2.3) based on the principal that the chlorophyll present in the leaf absorbs light with different wavelengths to different extents. Total chlorophyll content was recorded, by measuring four leaves from each plant.

SPAD meter is a simple, portable diagnostic tool for measurement of relative chlorophyll concentration, or greenness, of leaves. The SPAD values are positively correlated with the content of chlorophyll and they provide reliable estimate to the level of chlorophyll.

The extent of chlorophyll light quenching is closely correlated with the leaf chlorophyll content. The light absorbance maximum of the chlorophyll molecules is found in the blue and red wavelength ranges, while quenching is low in the green and yellow range and nearly zero in the infrared range.

The infrared range can thus be used as a reference value, while measurements are made in the blue and red range. The SPAD-502 takes readings in red light as the absorbance of this wavelength is not influenced by the leaf carotene content. The calculation of chlorophyll content is based on the ratio of the intensity of infrared and red light passing through the leaf. This ratio increases with the quantity of red light absorbed by the leaf which is in close correlation with the chlorophyll content.

The meter makes instantaneous and non-destructive readings on a plant based on the quantification of light intensity (peak wavelength: approximately 650 nm, red LED) absorbed by the tissue sample. A second peak (peak wavelength: 940 nm, infrared LED) is emitted simultaneous with red LED to compensate for the thickness of the leaf (Nikon Camera Co. Ltd., 2007). SPAD values range from 0-100.

Measurements were obtained once a week for the duration of the experiment.



Figure 2.3. SPAD-502 apparatus (Minolta Co. Ltd., Japan). Chlorophyll content was recorded, by measuring four leaves from each plant

2.2.3 Chlorophyll Fluorescence

The same leaves used for estimation of chlorophyll content above, were also used for chlorophyll fluorescence measurements. Chlorophyll fluorescence of the leaves was measured using a Chlorophyll Fluorometer (OS-30P, Opti-Sciences) after 15 min dark acclimation (Fig. 2.4). The following parameters were determined:

- i) Minimal fluorescence (arbitrary units) F_0 , fluorescence occurring when all antenna sites are assumed to be open (dark adapted)
- ii) Maximal fluorescence (arbitrary units) F_m , fluorescence intensity under exposure to saturation flash. All antenna sites are assumed to be closed
- iii) Terminal fluorescence, F_{tr} , fluorescence quenching value at the end of the test

iv) $T1 / 2$ (mS or μ S), half rise time from F_0 to F_m .

Also, the ratio of variable fluorescence to maximal fluorescence, F_v/F_m , calculated as $F_v / F_m = (F_m - F_0) / F_m$, variable fluorescence (F_v) is the change in fluorescence due to initial electron backup in the antenna complex.

Measurements were obtained once a week for the duration of the experiment.



Figure 2.4. Chlorophyll fluorescence of the leaves was measured using a Chlorophyll Fluorometer (OS-30P, Opti-Sciences)

2.2.4 Net Photosynthesis Rate

The net photosynthesis rate (P_n), was measured, using a portable infrared gas analyzer (LI-COR, LI-6400) (Fig. 2.5). Net photosynthesis was recorded for selected leaves of various developmental stages. The gas analyzer was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Measurements were taken at ambient temperature and humidity and with a light intensity $1,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$, CO_2 was maintained at a constant level of $400 \mu\text{mol L}^{-1}$ using an LI-6400-01 CO_2 injector with a high pressure liquefied CO_2 cartridge source. Measurements were obtained once a week for the duration of the experiment.



Figure 2.5. Portable infrared gas analyzer (LI-COR, LI-6400)

2.2.5 Proline Content

Proline concentration was determined according to Bates et al. (1973). A 0.2 g sample of leaf tissue was pulverized using liquid nitrogen and extracted in 1 mL TCA 10% w/v. The extracts were centrifuged at 2,000g for 15 min. Following, 300 μ L of the supernatant were treated with 300 μ L of glacial acetic acid and 300 μ L of ninhydrin solution and then, boiled at 95°C for 60 min. Proline content was measured at 546 nm by using a UV/VIS spectrophotometer (UV mini 1240, Shimadzu, Japan) and calculated as $\mu\text{mol g}^{-1}$ FW.

Ninhydrin solution contained: 2.5% w/v ninhydrin, in solution of acetic acid, H₂O and orthophosphoric acid (CH₃COOH: H₂O: H₃O₄P, 6:3:1 v/v).

2.2.6 Evaluation of Antioxidant Activity (TEAC – Trolox Equivalent Antioxidant Capacity)

The TEAC (Trolox (6-hydroxy-2, 5, 7, 8 – tetramethylchroman - 2 - carboxylic acid) equivalent antioxidant capacity) assay is based on scavenging of 2,2'-azinobis-(3-

ethylbenzothiazoline-6-sulfonate) radical anions (ABTS⁻). Because of difficulty in measuring each antioxidant component separately and interactions among antioxidants, methods have been developed to assess the total antioxidant capacity. The Trolox-equivalent antioxidant capacity (TEAC) assay is commonly used and has been extensively evaluated.

The method is based upon the controlled oxidation of a substance ABTS or 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). ABTS radical cation (ABTS⁺) is obtained from ABTS by oxidation and has a characteristic blue-green colour which is measured with a spectrophotometer at 645, 734, and 815 nm. The 734 nm wavelength has been adopted by most researchers to decrease interference from other components and minimize sample turbidity (Antolovich et al., 2002).

The presence of antioxidants in the sample inhibits the production of this colour in proportion to their concentration. So, by controlling the discoloration of the ABTS⁺ in solution, after the addition of the antioxidant, the activity of the latter can be measured.

The TEAC test was done with a modified method of TEAC (Muhammad et al., 2008; Ozgen et al., 2006). ABTS was dissolved in distilled water to a 7 mM concentration. ABTS⁺ was produced by reacting ABTS stock solution with 2.45 mM K₂S₂O₈ in a 20 mM Sodium acetate solution (CH₃COONa) and pH 4.5 allowing the mixture to stand, in the dark, at room temperature for 12 hours before the first use.

For study of antioxidant activity of standard antioxidant and plasma samples, the ABTS stock solution was diluted with PBS buffer (pH 7.4) to an absorbance of 1.2 ± 0.02 at 745 nm respectively, and equilibrated at 30 °C. A quantity of 0.2 g of the test sample was extracted with 2 mL aqueous 80% v/v acetone solution, and the extract was centrifuged at 2,000g for 10 minutes. For herbal extracts, dilutions were made in the respective organic solvents with which they had been extracted from aqueous solutions.

After addition of 10 µL of supernatant to 1 mL of diluted ABTS⁺ solution ($A = 1.2 \pm 0.02$), the absorbance was measured, within exactly 1 min intervals for 8 min. Solvent blanks were run in each assay for accurate readings.

All determinations were carried out at least three times in succession and in triplicate at each separate concentration level of the standards.

The percentage inhibition of absorbance at 734 nm was calculated by the following formula:

$$\% \text{ inhibition (at 734 nm)} = \left(1 - \frac{A_f}{A_o}\right) \times 100$$

A_o : the absorbance of radical cation solution before addition of sample/standard antioxidants.

A_f is the absorbance after addition of the sample/standard antioxidants.

The resultant data was plotted between concentration of antioxidants and that of trolox for the standard reference curve.

2.2.7 Evaluation of Lipid Peroxidation with the Method of Thiobarbituric Acid (TBARS)

The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to death of the cells. Lipid peroxidation refers to the oxidative degradation of lipids. It has been demonstrated that salt treatment increases lipid peroxidation or induces oxidative stress in plant tissues.

The thiobarbituric acid (TBA) reaction, quantified by colorimetry or fluorimetry, is the method most widely used for studying lipid peroxidation in laboratory. The methods used in the present study is based on the reaction of thiobarbituric acid (TBA) with aldehyde derivatives in lipid peroxidation at 100°C and is characterized as non-specific and calculates the total of all aldehyde derivatives (protein and non-protein) which are called TBARS (reacted with TBA, or TBA reactive substances) (Zamora et al., 1997).

The procedure of the method is shown below (Baryla et al., 2000):

Liquid nitrogen was used to grind 0.2g of sample, which was then, homogenized in 1 mL Trichloro Acetic Acid (TCA) 0.5% w/v, placed in a 2 mL tube and centrifuged for 15 minutes at 2,000 g. From the supernatant obtained, 300 μ L were transferred to a new tube with 1 mL 20% TCA with 0.5% TBA. The mixture was heated in a water bath at 95°C for 30 min. The samples were placed on ice for 5 minutes and the absorbance was measured at 532 nm and 600 nm.

The (malondialdehyde) MDA concentration was calculated as the difference in absorbance at 532 and 600 nm using the following formula:

$$\text{MDA level } (\mu\text{moles}) = A_{532 \text{ nm}} - A_{600 \text{ nm}}.$$

MDA was calculated from its molar extinction coefficient 156 $\mu\text{mol}^{-1}\text{cm}^{-1}$ and expressed as $\mu\text{moles/g}$

The conversion of absorbance to concentration was carried out by using a standard curve made with MDA manufactured following the same procedure.

2.2.8 Measurements of Soil pH and Electrical Conductivity (EC)

Soil salinity of saline soil have been determined using electrical conductivity for more than 100 years. Early methods included measurements directly on a soil paste, but the influence of the soil in the paste during the measurement was not fully understood until recently, leading to uncertainty in the measurements. By about 1940 the accepted method for determining soil salinity was to make a saturated paste by a specified procedure, extract solution from the paste, and measure the electrical conductivity of the solution (Richards, 1954). Soil is mixed with deionised water until a homogenous paste is formed. The paste is then centrifuged at 2,000 rpm for 15 min and the supernatant transferred to a clean container. The supernatant is then filtered through a cellulose filter. The filtered supernatant is then used for determination of pH with a ph meter (InoLAB pH level 2, WTW, Germany) and electrical conductivity, determined by an EC meter (Consort, C830). The measurement is referred to as the electrical conductivity of the saturation extract. These values were then correlated with crop response.

2.3 Greenhouse Experiment

Tomato seedlings, were commercially obtained and transferred to pots approximately 5 L in volume. Each pot was filled uniformly with 5 L homogenous Potgrond P (Klasmann-Deeilmann, German) substrate that contained blend of black sphagnum peat. The seedlings were grown in a greenhouse (Fig. 2.6) for a period of 4 weeks prior to treatments, during which they were irrigated every day.

At the end of this period, when plants were approximately 70 cm in height, treatments were carried out using 2 concentration of NaCl, 70 mM “NaCl1” and 140 mM “NaCl2”, 4 different concentrations of Milsana (0.0 - 0.5 – 1.0 – 2.0% v/v) and combinations of the above (NaCl1+0.5% Milsana – NaCl1+1.0% Milsana – NaCl1+2.0%Milsana, NaCl2+0.5% Milsana – NaCl2 + 1.0%Milsana – NaCl2+2.0%Milsana).

Plants irrigated and sprayed with distilled H₂O were used as controls. Plants were irrigated once weekly with a solution of NaCl and Milsana by spraying on leaves, once a week for a period of 13 weeks (91 days) (10 replicates per treatment). Time points and intensity of treatments were selected according to preliminary experiments.



Figure 2.6: Tomato seedlings (*Solanum lycopersicum*) grown in greenhouse

2.3.1 Morphological Characteristics and Growth Parameters

Total tomato fruit number and height of plants was measured to provide an estimate of growth for the treated and control plants. The weight and the number of tomato fruits was measured per plant and per treatment. Measurements were obtained twice for the duration of the experiment.

The fresh weight of the above ground part of tomato plants was determined, and so was the dry weight after drying in an oven at 70 ° C for 30 hours. The morphological characteristics of tomato plants were monitored for the duration of the experimental period and the symptoms were observed.

2.3.2 Physiological and Photochemical Parameters - Chemical and Biochemical Analyses

The physiological (net photosynthesis rate) and photochemical parameters (chlorophyll content and chlorophyll fluorescence measurements) as well as the chemical and biochemical analyses (proline content, evaluation of antioxidant capacity and the evaluation of lipid peroxidation) were measured as described above in the growth chamber experiment section.

Proline content, evaluation of antioxidant capacity and the evaluation of lipid peroxidation were measured on upper (the youngest fully expanded leaf near the tip) and lower (3rd-4th leaves from the base) leaves of tomato plants.

2.3.3 Fruit Colour Measurements

Colour of tomato fruits is used to determine ripeness as the fruits change colour during the ripening process. On harvest day, 10 fruit of each treatment were used to measure chromaticity values. Measurements of the chromaticity values were taken on whole fruit

and puree of tomato. Fruit colour and puree of tomato was measured using a Konica Minolta CR 400 Colorimeter (Konica Minolta) (Fig. 2.7), expressing colour change.

The colour variation was measured directly on the fruit. The colour was recorded using L^* (lightness), C^* (chroma), and h^* (hue angle) values. The L^* value is the measurement of the lightness or the amount of light reflected. The C^* and h^* values are measures of the chroma and hue angle, respectively. The L^* corresponds to values from 0 to 100 where 0 refers to black and 100 to white. The C^* value refers to colour density. The h^* value is measured in degrees and determines the hue where 0° refers to red, 90° refers to yellow, 180° refers to blue green and the 270° value refers to blue (Holcroft and Kader, 1999).

Colour or pigment changes during tomato ripening are characterized by loss of chlorophyll and a rapid accumulation of carotenoids, particularly lycopene. Lycopene is the predominant pigment in tomato that imparts the attractive redness.



Figure 2.7: Konica Minolta CR 400 Colorimeter

2.3.4 Measurements of °BRIX (Total Soluble Solids) in Tomato Juice Samples

Tomato juice samples were used to measure °Brix (total soluble solids) using a BrixMeter™ (a.k.a. refractometer). This meter works on the principle of light bending

when it passes from air into water. Due to dissolved sugars, minerals and other nutrients in fruit and vegetable juices, they are denser than water and bend light more. The amount light bends is measured in degrees Brix ($^{\circ}\text{Brix}$).

Degrees Brix ($^{\circ}\text{Brix}$) are the total soluble solids of a juice solution. One degree Brix corresponds to 1 g of sucrose in 100 g of solution and represents the strength of the solution % w/w of soluble solids. A few drops of tomato juice (drops from several fruits per treatment) were added to the prism and it was held up to a light source.

The light is refracted as it passes through the liquid and measurements correspond to a Brix scale. As the soluble solids content of the juice goes higher, so does the indication of the Brix meter (Fig. 2.8).



Figure 2.8: Brix meter

2.3.5 Firmness of Whole Mature Tomato Fruit

The firmness of whole mature tomato fruits was determined by measuring the force necessary to penetrate the fruit. In order to determine the penetration force, we used a Chatillon Mod. DPP Dial Push-Pull Gauge (Fig. 2.9) with a cylindrical needle of 8 mm diameter and 1 mm height. The needle penetrated the fruit vertically until the needle reached an indicated point.



Figure 2.9: Chatillon Mod. DPP Dial Push-Pull Gauge

2.3.6 Statistical Analysis

The experiment plan was randomized. For the physiological and photochemical parameters, 10 measurements were taken, while for the biochemical parameters we took 3 measurements per treatment. The results are presented as mean values $\pm$ the standard error of the mean.

The statistical analysis of the data was performed using of Analysis of Variance (ANOVA) at a significance level of $p=0.05$, and applying the Bonferroni correction. All the aforementioned analyses were carried out using GraphPad Prism ver.11 software. Values which differed significantly from one another are marked with a different letter (Duncan, $p=0.05$).

CHAPTER 3

Results and Discussion

3.1 Growth Chamber Experiment

Tomato plants were grown in the growth chamber (Fig. 3.1) and irrigated once a week for a period of 9 weeks. Treatments were carried out using two levels of NaCl, (70 mM and 140 mM, “NaCl1” “NaCl2”) and 4 different concentrations of Milsana, (0.0% (Control), 0.5% (M1) – 1.0% (M2)– 2.0% (M3) v/v), and combinations of the above (NaCl1 -M1, NaCl1 - M2, NaCl1 - M3, NaCl2 - M1, NaCl2 - M2, NaCl2 - M3). Plants irrigated and sprayed on, with distilled H₂O, were used as controls.

Plants were irrigated once weekly with a solution of NaCl and Milsana was applied, by spraying on leaves, once a week for 9 weeks. Time points and intensity of treatments were selected according to preliminary experiments. During that period, several parameters related to plant growth were monitored. At the end of the experiment, leaf tissue samples were harvested and used for further analysis. Data for the parameters followed and presented in tables and figures.



Figure 3.1: General view of tomato plants cultured grown in the growth chamber.

3.1.1 Substrate pH and EC

At the end of the experiment substrate pH and EC were measured. The pH of the growing medium varied between 4.1 and 4.3 across all treatments (Table 3.1). In the control (0.0 mM NaCl) and Milsana treatments, the substrate showed the same EC values. The addition of 70 mM NaCl and 140 mM NaCl, led to 1-fold and 4-fold increase in EC values respectively, compared to the control plants (Table 3.1). In contrast pH values were similar across all treatments. Our observations agree with the experiments of Mitchell et al. (1991), who recorded an increase of EC values in NaCl treated tomato plants compared to untreated plants.

Table 3.1: The pH and electrical conductivity (EC) values of the growing substrates of treated or un-treated plants at the end of the experiment. Each value is a mean of 3 replications. Values which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

Treatments	pH	EC (mS/cm)
Control	4.3a	3.4a
M 1	4.3a	3.3a
M 2	4.3a	3.6a
M 3	4.3a	3.4a
NaCl 1	4.2a	8.4b
NaCl 1 – M 1	4.2a	9.6b
NaCl 1 – M 2	4.1a	8.3b
NaCl 1 – M 3	4.1a	8.9b
NaCl 2	4.2a	14.6c
NaCl 2 – M 1	4.3a	12.7c
NaCl 2 – M 2	4.2a	14.6c
NaCl 2 – M 3	4.1a	15.0c

3.1.2 Effect of concentration of NaCl and Milsana Treatments on Growth Parameters of Tomato Plants

The above ground height of treated and control plants was measured to the level of the meristem. Interestingly, application of 2.0% Milsana to plants irrigated with plain water (0mM NaCl) led to changes in growth, in contrast to the rest of Milsana treatments (Fig. 3.2). Overall, 2.0% Milsana had the most positive effect on plant height, and plants treated with this concentration had the greatest increase in height, compared to the control, 0.5% and 1.0% Milsana, treated plants (Fig. 3.2, 3.3). In contrast, treatments with NaCl and combined treatments (NaCl + Milsana) did not lead to any significant alterations in plant height (Fig. 3.4, 3.5).

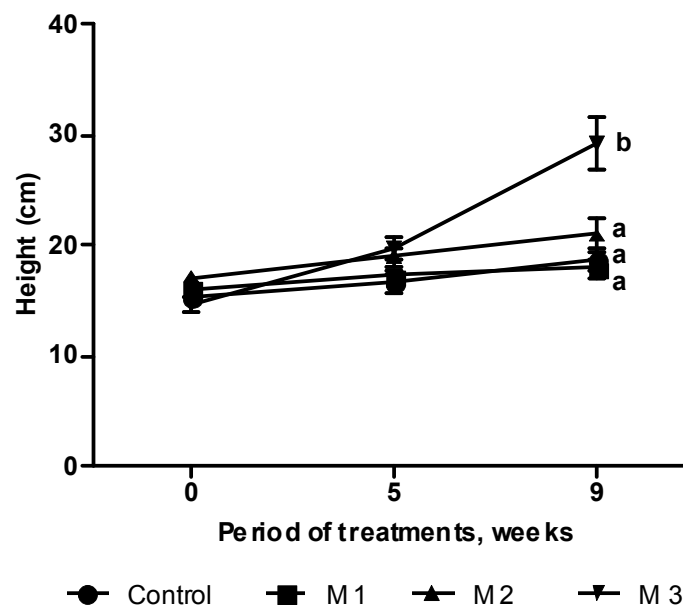


Figure 3.2: Effect of Milsana (0mM NaCl) on tomato plant height. Plants were treated with 0.0 (Control), 0.5 (M1), 1.0 (M2) or 2.0 % v/v (M3) Milsana by spraying once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$)



Figure 3.3: Plants of tomatoes grown in growth chamber, conditions in the presence of 0.0, 0.5, 1.0, 2.0 v/v Milsana

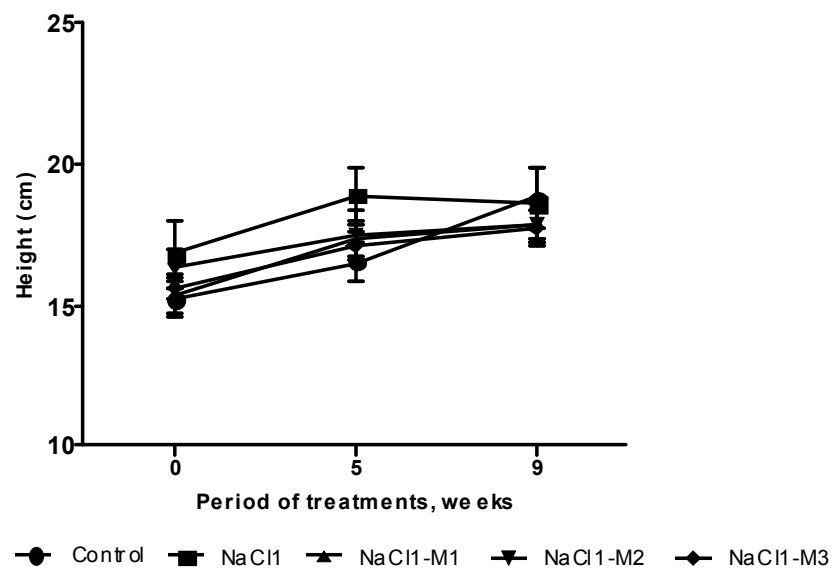


Figure 3.4: Effect of Milsana on the growth of tomato plants under NaCl stress. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 10 repetitions.

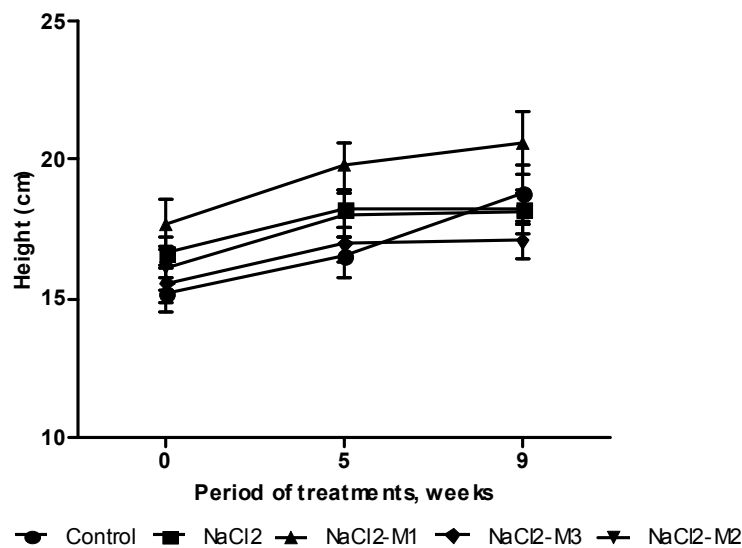


Figure 3.5: Effect of Milsana on the growth of tomato plants under NaCl stress. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 10 repetitions.

3.1.3 Effect of Concentration of NaCl and Milsana on Photosynthetic Parameters on Tomato Leaves

3.1.3.1 Chlorophyll content

Photosynthesis is among the primary processes to be affected by salinity. The effects can be direct, as in the form of decreased CO₂ availability or they can arise as secondary effects, namely oxidative stress. The latter are mostly present under multiple stress conditions and can seriously affect leaf photosynthetic machinery (Chaves et al., 2009).

To further test the ability of plants to carry out photosynthesis, the chlorophyll content was determined in NaCl and Milsana treated plants. Interestingly the chlorophyll content of Milsana only treated plants was similar to the chlorophyll content of control plants, for the duration of the test (Fig. 3.6).

According to Schmitt (2002), in cucumber plants, treated with extract of *R. sachalinensis*, the chlorophyll content was significantly increased from 0.34 (control) to 0.52 mg/g fresh weight (extract treatment). In other experiments, chlorophyll content of tomato plants treated with Milsana, did not statistically differ among treatments (Bouchagier et al., 2009).

In this study plants treated with 70mM NaCl + 0.5% Milsana had the highest chlorophyll content, compared to all other treated (70mM NaCl, combined of 70mM NaCl+ 1.0% and 2.0% Milsana) and untreated plants (Fig. 3.7). The chlorophyll contents of the combination of 0.5% 1.0% and 2.0% Milsana with 140mM NaCl, were significantly higher, compared to the 140 mM NaCl treated plant (Fig. 3.8).

The 140mM NaCl treated plants had a low chlorophyll content for the duration of the experiment, compared to all treated and untreated plants which suggest that there might have been some damage due to salinity to photosystem II reaction centres, or, in the transfer of excitation energy from the antenna to the reaction centres (Dadkhah, 2011).

Overall, the combination of 70mM and 140 mM NaCl and Milsana treated plants (N1-M1,2,3 and N2-M1,2,3) led to an increase in chlorophyll content, compared to the NaCl treatments (N1, N2) (Fig. 3.7, 3.8).

A decrease in the chlorophyll content under salinity stress has been reported by several workers. The chlorophyll content on pepper leaves was reduced due to high salinity (75 and 100 mM NaCl)(Günes et al., 1996).

Decreases in chlorophyll content in response to salt stress is a general phenomenon which leads to a disordering in the synthesis of chlorophyll and development of chlorosis in plants (Parida and Das, 2005). Ali et al. (2004) attributed this reduction in chlorophyll concentration by NaCl to the inhibitory effect of the accumulated ions of various salts on the biosynthesis of the different chlorophyll fractions.

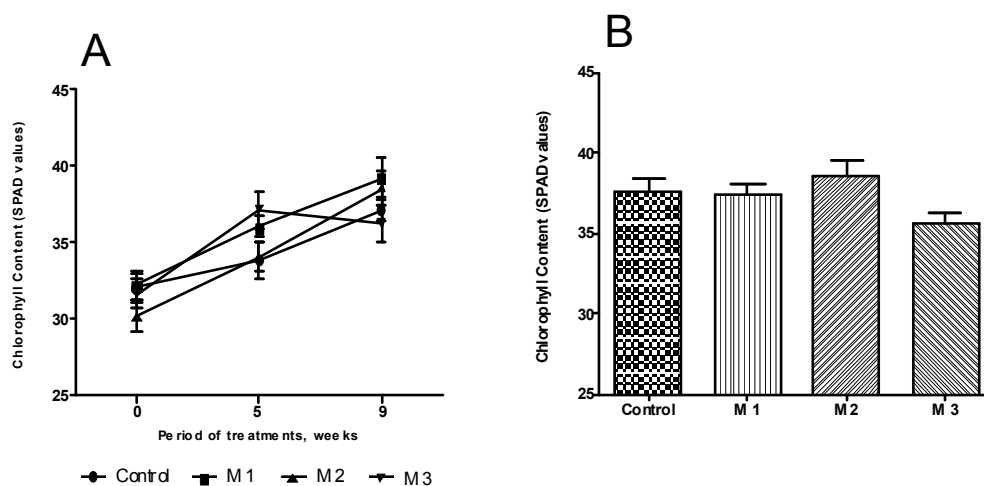


Figure 3.6: Effect of Milsana on chlorophyll content (SPAD values) of tomato leaves. A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week. Values are means $\pm$ standard error (SE) of 10 repetitions.

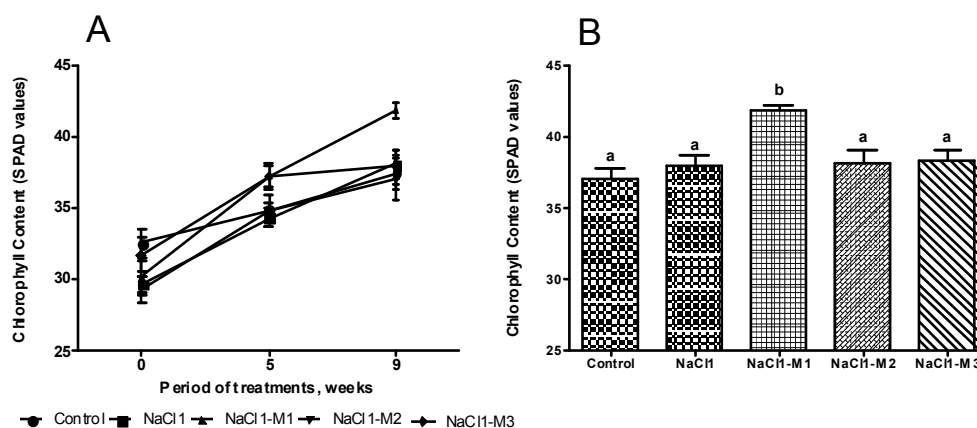


Figure 3.7: Effect of Milsana on chlorophyll content (SPAD values) of tomato leaves under NaCl stress, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

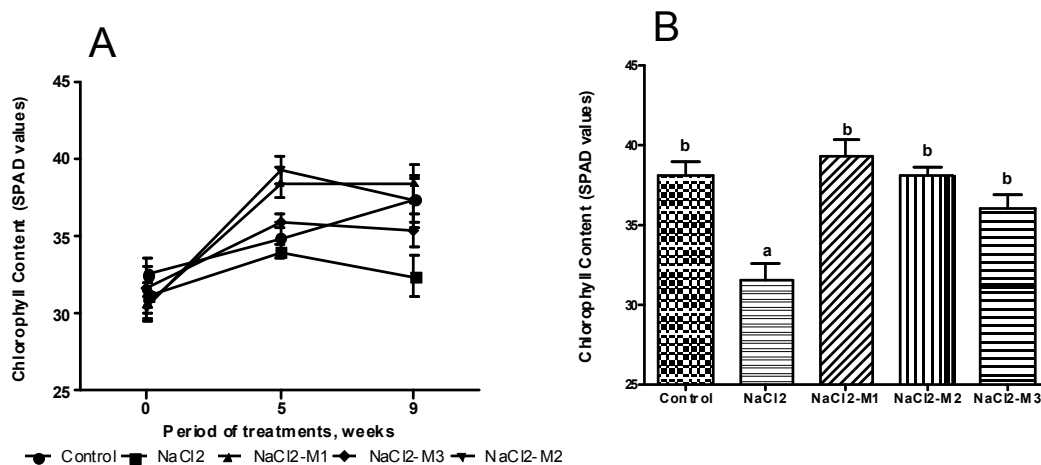


Figure 3.8: Effect of Milsana on chlorophyll content (SPAD values) of tomato leaves under NaCl stress, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.1.3.2 Chlorophyll Fluorescence

The ratio of F_v/F_m is a dark-adapted test used to determine maximum quantum yield. Therefore it can be used as an estimate of the efficiency of the photosynthetic machinery and the impact of stress on it. Higher values are representative of “healthier” photosynthetic machinery, whereas lower values are an indication of photosynthesis malfunction and stress. The F_v/F_m of 0.5%, 1.0% and 2.0% Milsana, treated plants, remained fairly unchanged, compared to untreated plants (Fig. 3.9). The combination of treatment with 70mM NaCl and Milsana did not led to a change in the F_v/F_m values of treated plants, compared to the other treatments (70mM NaCl-N1) and the control plants (Fig. 3.10). The F_v/F_m ratio of plants treated with the combination of 140mM NaCl and 0.5% Milsana was increased compared to the plants treated with 140mM NaCl (N2) and were similar to the ratios determined for control plants. The 140mM NaCl treated plants scored low for F_v/F_m for the duration of the experiment, compared to control plants (Fig. 3.11). Interestingly the combined treatments maintained a ratio similar to the one determined for control plants, in contrast to NaCl treated plants, which scored lower

than the untreated plants. In other experiments, a significant reduction in Fv/Fm in NaCl treated plants was observed, which was attributed to the non-stomatal inhibition of photosynthesis, caused by direct effects of NaCl on photosynthetic apparatus (Kao et al., 2003).

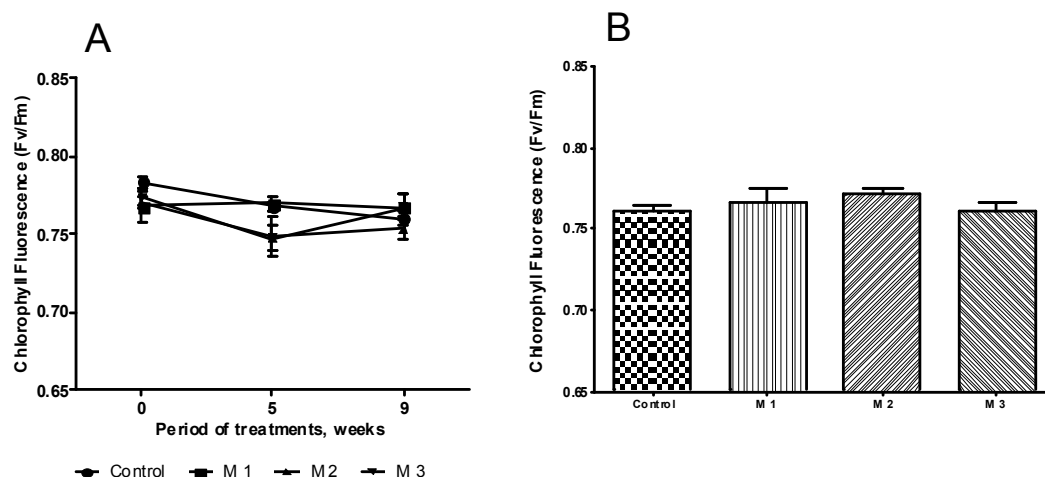


Figure 3.9: Effect of Milsana on Chlorophyll Fluorescence (Fv/Fm) of tomato leaves, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week. Values are means $\pm$ standard error (SE) of 10 repetitions.

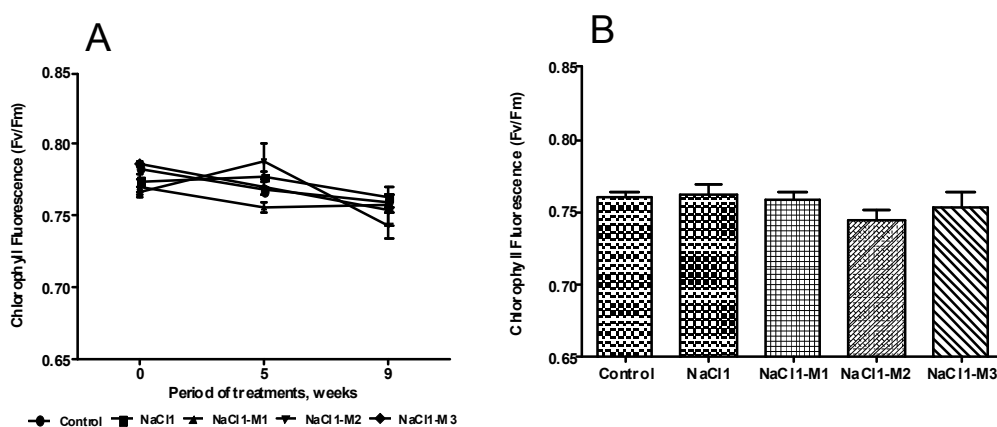


Figure 3.10: Effect of Milsana on Chlorophyll Fluorescence (Fv/Fm) of tomato leaves under NaCl stress, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions.

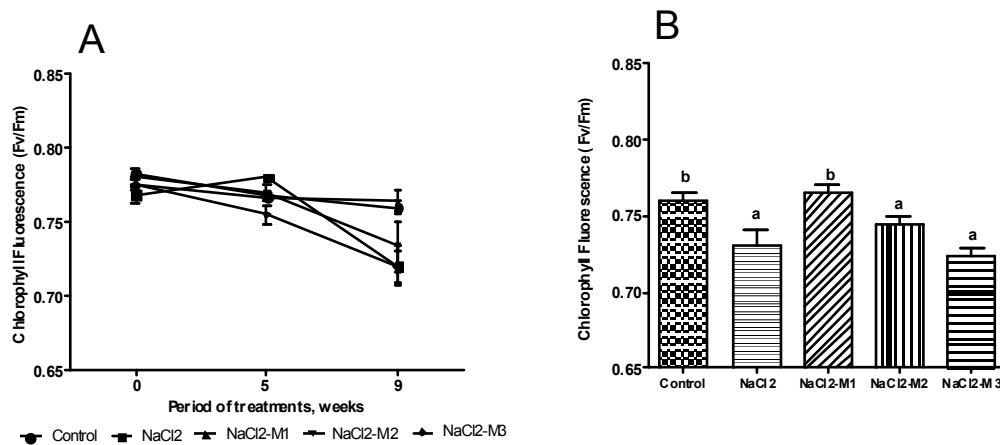


Figure 3.11: Effect of Milsana on Chlorophyll Fluorescence (Fv/Fm) of tomato leaves under NaCl stress, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.1.3.3 Photosynthesis Rate

Leaf photosynthetic rates (P_n) of 0.5, 1.0, 2.0 % Milsana treated plants were similar to the rates determined for control plants (Fig. 3.12). The lowest rates were observed in the 70 and 140mM NaCl treated plants. The photosynthetic rates of the combined 70mM NaCl + 0.5% or 1.0% Milsana treatments were similar to the ones determined for the 70mM treated plants. Interestingly, the combined treatments with 70mM NaCl + 2.0% Milsana had higher leaf photosynthetic rates compared to 70mM NaCl treated plants at the final week of the experiment (Fig. 3.13). Finally the combined treatment of 140mM NaCl + 0.5 or 1.0% Milsana exhibited a higher photosynthetic rate compared to 140mM NaCl treated plants in the final week. It is also worth mentioning that the photosynthetic rate of the 140mM NaCl treated plants was lower in the final week compared to the rates determined for all treated and untreated plants (Fig. 3.14).

According to Chartzoulakis et al. (1995), reduced rates of photosynthesis in salt stressed plants can be attributed in part to stomatal closure upon salinization and to the effects of salts on the capacity of the plant for CO₂ fixation. All photosynthetic parameters were

significantly decreased by increasing salinity. The present study indicated substantially lower rates of net photosynthesis with increasing salinity. It was shown that treatments of untreated plants (0mM NaCl) with Milsana favourably influenced the photosynthetic apparatus of the plants and stimulated photosynthetic activity of the tomato plants.

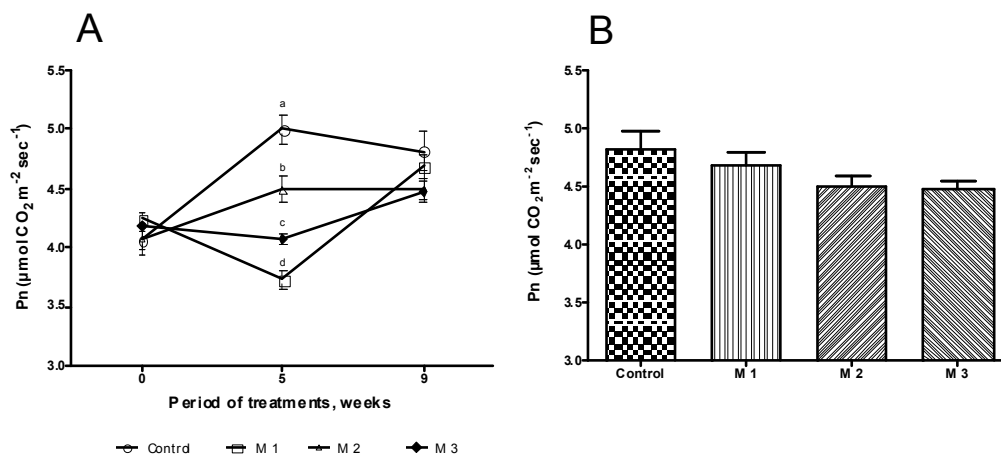


Figure 3.12: Effect of Milsana on Photosynthesis rate (Pn) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}$) of tomato leaves, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week. Values are means $\pm$ standard error (SE) of 10 repetitions.

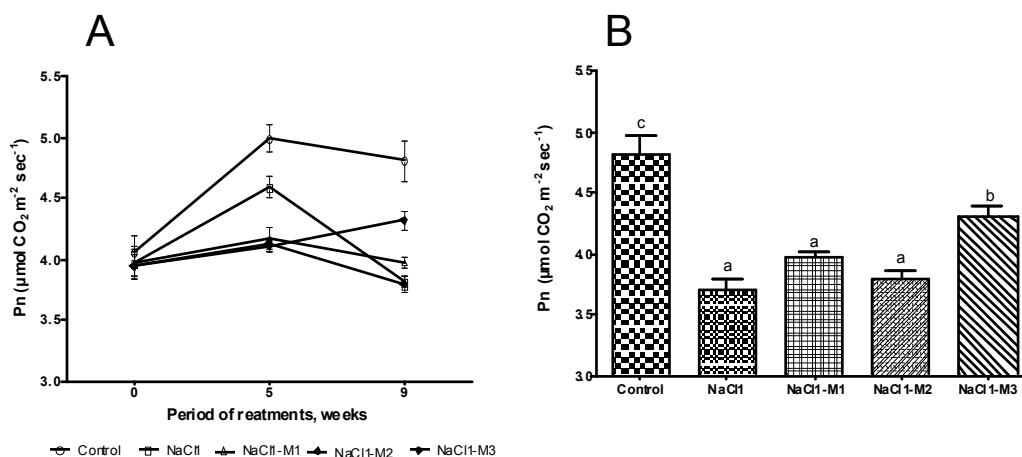


Figure 3.13: Effect of Milsana on Photosynthesis rate (Pn) of tomato leaves under NaCl stress, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

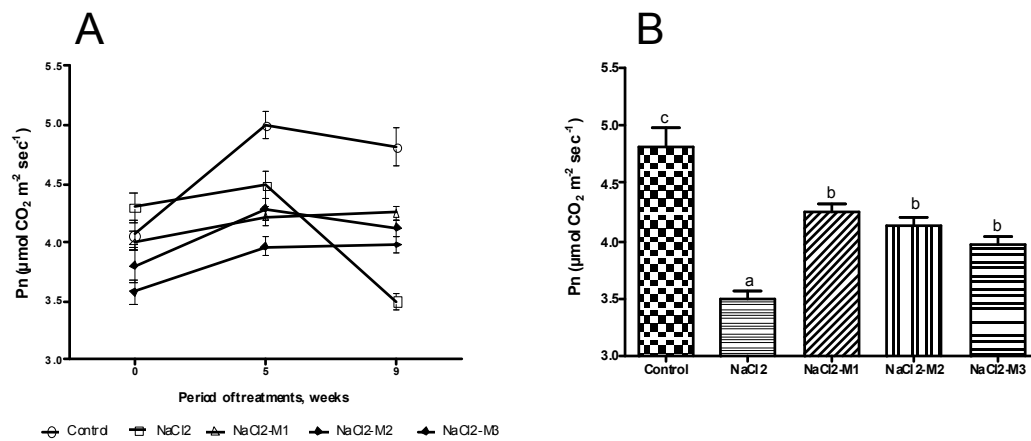


Figure 3.14: Effect of Milsana on Photosynthesis rate (Pn) of tomato leaves under NaCl stress, A) for the duration of the treatments (9 weeks) B) during the final week (9th week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.1.4 Effect of Milsana Treatments on the Antioxidative Machinery under NaCl Stress

3.1.4.1 Proline content

Salt stress results in a wide variety of physiological and biochemical changes in plants. Proline is an amino acid, which acts as an organic solute and plays a major role in this osmotic adjustment of salt stressed plants. Biochemical analysis of tomato leaves and mature fruits for proline accumulation has shown that proline accumulation increases with increase in salt stress (Babu et al., 2012).

Treatments with Milsana did not lead to any significant increase compared the control plants in proline content (Fig. 3.15). Treatments with NaCl and combined treatments with NaCl and Milsana had the highest proline content when compared to untreated plants (Fig. 3.16, 3.17).

Overall salinity increased the proline contents in tomato leaves. In general, amino acids, such as proline, are related in their metabolic pathways and affected by alteration in enzymatic levels, usually associated with salinity.

Our experiment showed that proline accumulation increased with increase in salt stress. These results agree with those of Babu et al. (2012). In contrast no significant changes were observed in proline content when Milsana was sprayed on leaves, in the presence, or absence of NaCl.

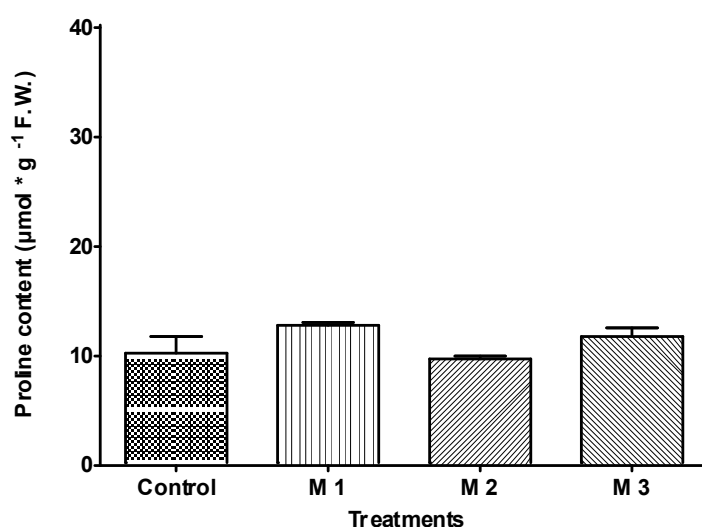


Figure 3.15: Effect of Milsana on Proline content of tomato leaves. Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions.

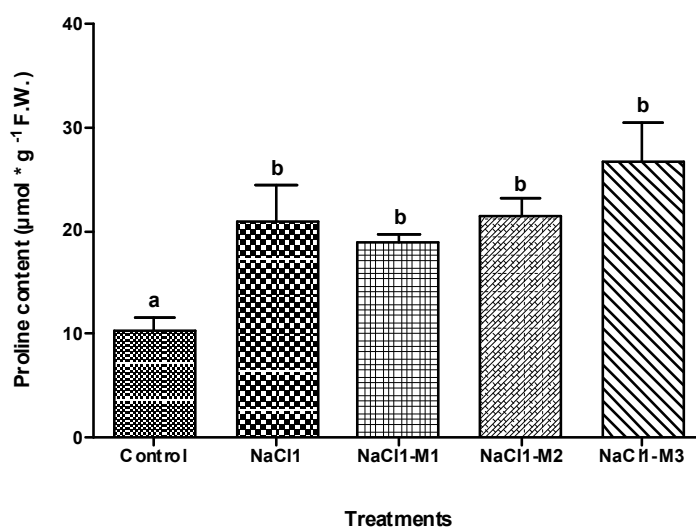


Figure 3.16: Effect of Milsana on Proline content of tomato leaves under NaCl stress. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

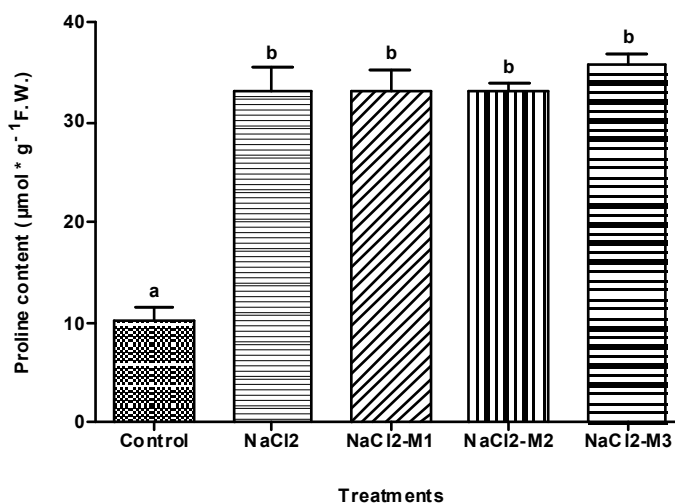


Figure 3.17: Effect of Milsana on Proline content of tomato leaves under NaCl stress. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.1.4.2 Malondialdehyde (MDA) equivalents

Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage. ROS cause peroxidation of polyunsaturated fatty acids, producing α,β -unsaturated aldehydes such as malondialdehyde (MDA).

These aldehydic secondary products of lipid peroxidation are generally accepted as markers of oxidative stress. It has also been demonstrated that salt treatment increases lipid peroxidation or induces oxidative stress in plant tissues (Shulaev and Oliver, 2006).

In this report Malondialdehyde (MDA) determination was used as an index of lipid peroxidation. Treatments with Milsana did not lead to any significant increase compared the the control plants in MDA equivalents (Fig. 3.18).

The combined treatment of 70 mM NaCl + 0.5 or 1.0% Milsana exhibited a higher MDA equivalent compared to 70 mM NaCl treated plants. It is also worth mentioning that the MDA equivalent of 70 Mm NaCl treated plants was lower compared to the rates determined for all treated and untreated plants (Fig. 3.19). The combined treatment of 140 mM NaCl +Milsana exhibited a lower MDA equivalent compared to 140 mM NaCl treated plants. The MDA equivalent of 140 mM NaCl treated plants was higher compared to the rates determined for all treated and untreated plants (Fig. 3.20).

Previous work showed that Milsana induces resistance in plants which is associated with the activation of plant defence responses, conferring to this product the status of a 'defence inducer'.

Our results are in agreement with current literature, where Milsana has been found to act on the wheat compatible interaction at several levels such as a slight decrease of the lipid peroxide level (Daayf et al., 1997).

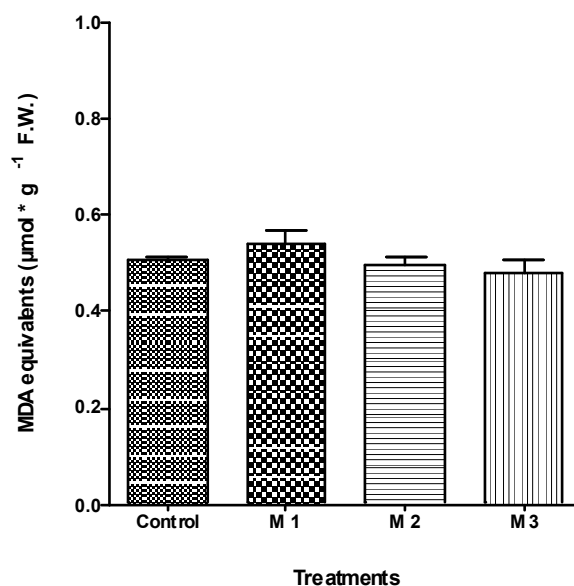


Figure 3.18: Effect of Milsana on Malondialdehyde (MDA) equivalents of tomato leaves. Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions.

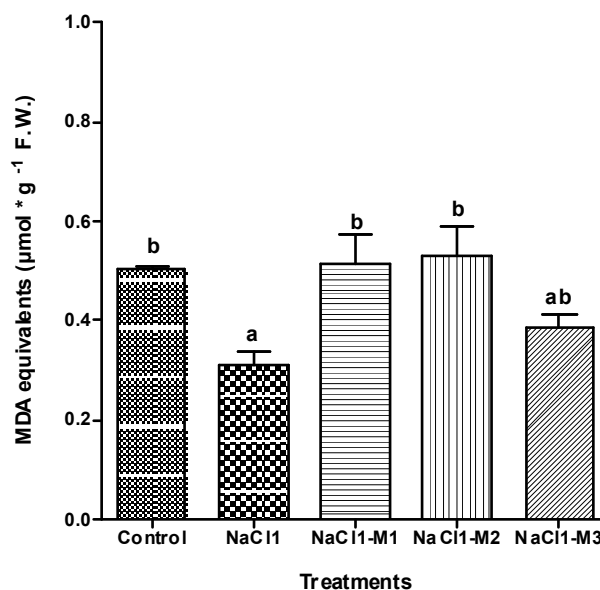


Figure 3.19: Effect of Milsana on Malondialdehyde (MDA) equivalents of tomato leaves under NaCl stress. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

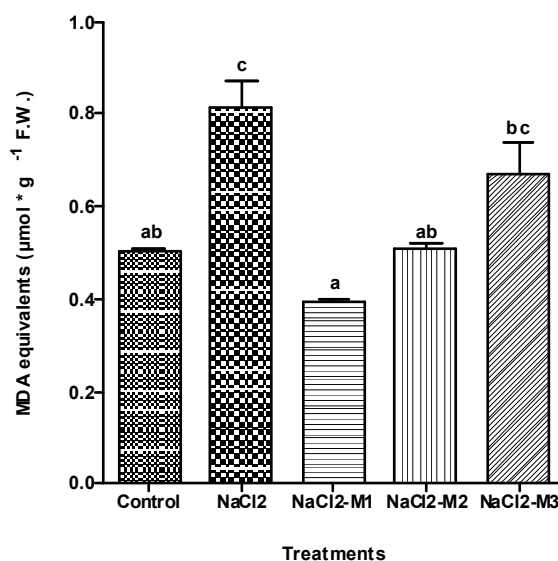


Figure 3.20: Effect of Milsana on Malondialdehyde (MDA) equivalents of tomato leaves under NaCl stress. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.1.4.3 Trolox equivalents Antioxidant capacity

Antioxidant activities were determined by TEAC (Trolox equivalent antioxidant capacity).

Treatments with Milsana or Milsana+70mM NaCl did not lead to any significant increase in Trolox equivalents antioxidant capacity compared to the control plants (Fig. 3.21). The combined treatment of 70mM NaCl + 2.0%Milsana exhibited a higher Trolox equivalent compared to 70mM NaCl treated plants (Fig. 3.22). Treatments with 140mM NaCl had the highest Trolox equivalents compared to all treated and untreated plants (Fig. 3.23).

According to Fofana et al. (2002) and Daayf et al. (1997), Milsana acts by enhancing the flavonoid metabolic pathway. Treatment of cucumber plants resulted in an increase in the levels of two genes responsible for the expression of two enzymes of the

flavonoid biosynthetic pathway, namely chalcone synthase (CS) and chalcone isomerase (CI) (Fofana et al., 2002).

Flavonoids are phenolic substances and act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening. Most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals (Pietta, 2000).

In contrast, when 140mM NaCl was added TEAC was dramatically enhanced suggesting a triggering salinity effect. Milsana spraying in 140mM NaCl treated plants significantly alleviated the TEAC enhancement, by keeping TEAC values comparable to control plants.

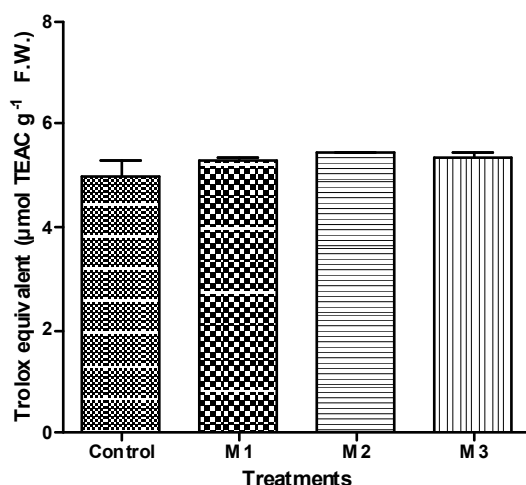


Figure 3.21: Effect of Milsana on Trolox equivalents Antioxidant capacity of tomato leaves. Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions.

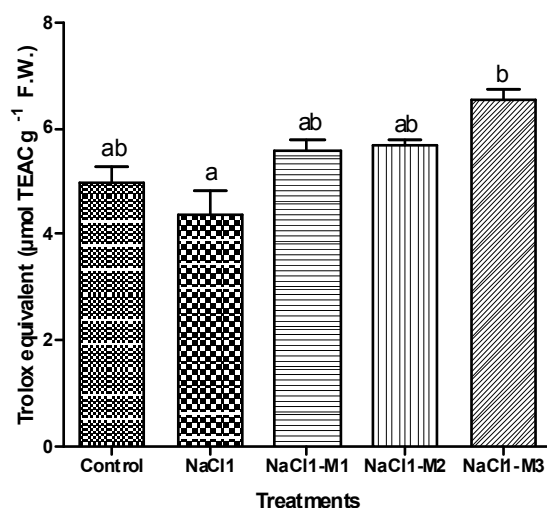


Figure 3.22: Effect of Milsana on Trolox equivalents Antioxidant capacity of tomato leaves under NaCl stress. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

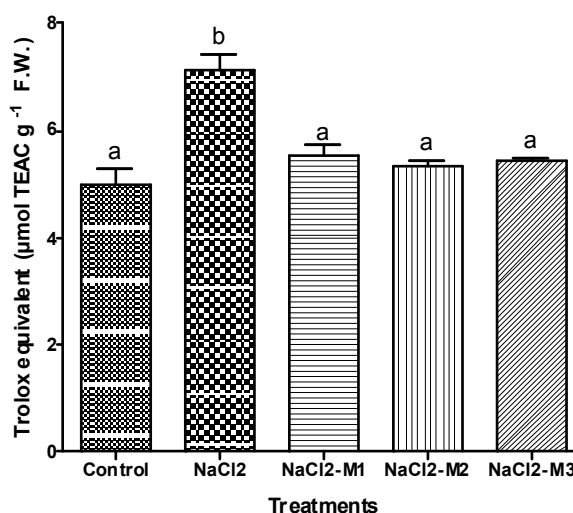


Figure 3.23: Effect of Milsana on Trolox equivalents Antioxidant capacity of tomato leaves under NaCl stress. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 9 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2 Greenhouse Experiment

Tomato seedlings (*Solanum lycopersicum*) were commercially obtained, and transferred to pots approximately 5 L in volume. The transferred plants were grown in the greenhouse (Fig. 3.24), for a period of 4 weeks prior to treatments, during which they were irrigated every day. Treatments were carried out using two concentrations of NaCl, 70mM “NaCl1” and 140 mM “NaCl2”, 3 different concentrations of Milsana (0.5 – 1.0 – 2.0% v/v) and combinations of the above (NaCl1+0.5% Milsana – NaCl1+1.0% Milsana – NaCl1+2.0%Milsana, NaCl2+0.5% Milsana – NaCl2 + 1.0%Milsana – NaCl2+0.%Milsana).

Plants irrigated and sprayed with distilled H₂O, were used as controls. Plants were irrigated once weekly with a solution of NaCl and Milsana was applied, by spraying on leaves, once a week for a period of 13 weeks (91 days).

During that period, several parameters related to plant growth were monitored. At the 7th and at the 13th week of the experiment, leaf tissue samples, from the upper (the youngest fully expanded leaf near the tip) and lower (3rd-4th leaves from the base) zone of the plants were collected and used for further analysis.



Figure 3.24: General view of tomato plants cultured in the greenhouse.

3.2.1 Effect of Milsana Treatments on Morphological Characteristics and Growth on Tomato Plants under NaCl Stress

3.2.1.1 Height

The growth parameter tested was plant height. The above ground height of treated and control plants was measured to the level of the meristem. Treatment with 0.5% Milsana led to changes in growth, in contrast to untreated plants (0.0% Milsana control plants) (Fig. 3.25). Overall Milsana had a positive effect on plant height, and plants treated with this concentration (0.5%) had the greatest increase in height, compared to the control (Fig. 3.25). In contrast, combined treatments (70mM NaCl + Milsana) did not lead to any significant increases in plant height compared to NaCl (N1) treated plants (Fig. 3.26). Plants treated with 140mM NaCl and those treated with a combination of Milsana and NaCl, had significantly lower height compared to control plants (Fig. 3.27).

Combined treatments with 0.5% Milsana + 140mM NaCl had a positive effect on plant height as compared to 140mM NaCl and combined treatments of 140mm NaCl with 1.0% or 2.0% Milsana (Fig. 3.26). Overall, Milsana, in both greenhouse and growth chamber experiments, positively affected the growth of tomato plants in terms of plants height. On the contrary, all NaCl treatments (with or without Milsana) significantly decreased plant height.

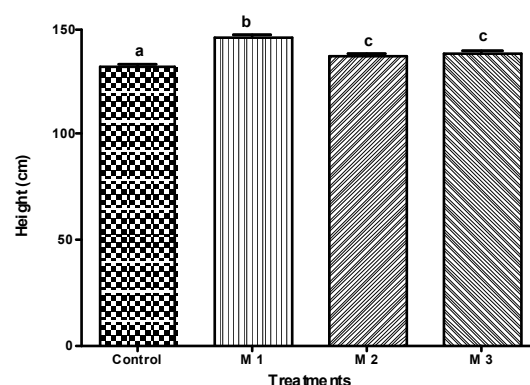


Figure 3.25: Effect of Milsana on tomato plant height (final week). Plants were treated with 0.0 (Control), 0.5 (M1), 1.0 (M2) or 2.0 % v/v (M3) Milsana by spraying once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

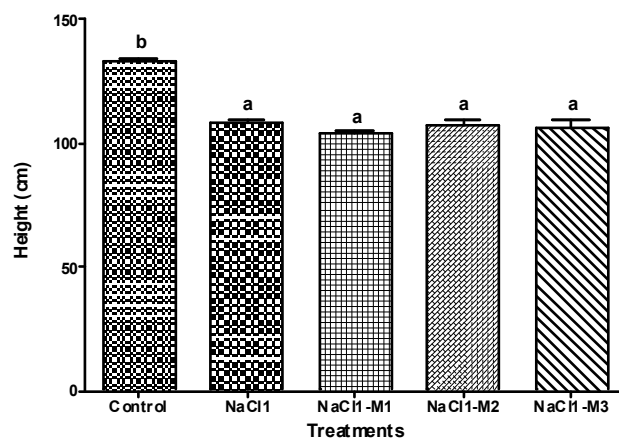


Figure 3.26: Effect of Milsana on tomato plant height under NaCl stress (final week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

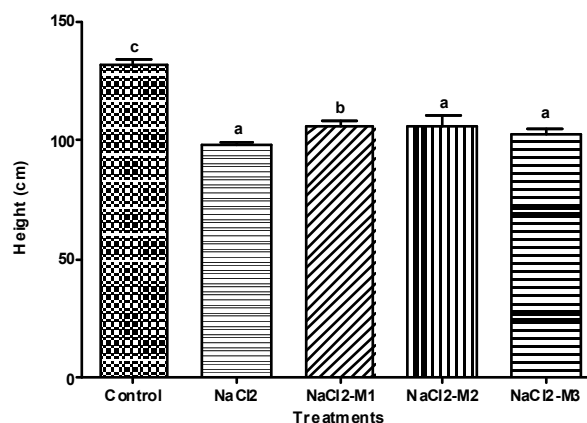


Figure 3.27: Effect of Milsana on tomato plant height under NaCl stress (final week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.2 Effect of Milsana Treatments on Photosynthetic Parameters on Tomato Leaves under NaCl Stress

3.2.2.1 Chlorophyll content

To further test the ability of plants to carry out photosynthesis, the chlorophyll content was determined on tomato leaves. Interestingly the chlorophyll content of 0.5 % Milsana (M1) treated plants was higher when compared to the chlorophyll content of control plants, for the duration of the test (Fig. 3.28). These results agree with those of Schmitt (2002). Plants treated with 70mM NaCl + 0.5% or 1.0% or 2.0% Milsana had the highest chlorophyll content, compared to 70mM NaCl treated plants (Fig. 3.29) suggesting a protective role of Milsana under salinity conditions. The chlorophyll content of the combination of 0.5% and 1.0% Milsana with 140mM NaCl was significantly higher, compared to the 140 mM NaCl treated plant. The 140mM NaCl treated plants low chlorophyll content for the duration of the experiment, compared to all treated and untreated plants (Fig. 3.30). As in the case of the growth chamber experiment Milsana application of NaCl⁻ stressed plants positively affected the chlorophyll content of the treated plants.

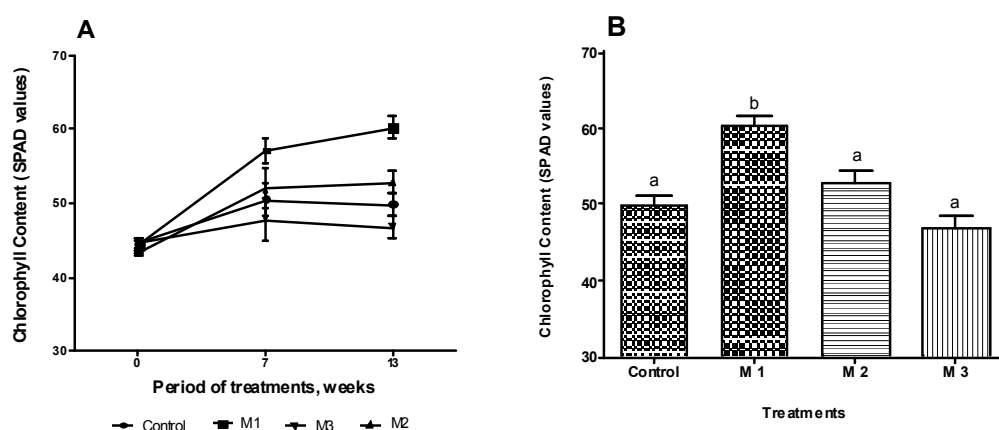


Figure 3.28: Effect of Milsana on Chlorophyll content (SPAD values) of tomato leaves, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

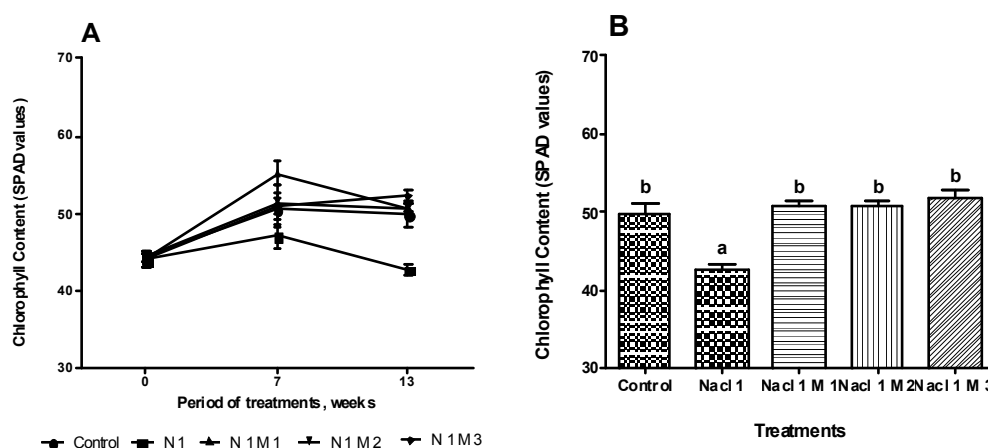


Figure 3.29: Effect of Milsana on Chlorophyll content (SPAD values) of tomato leaves under NaCl stress, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

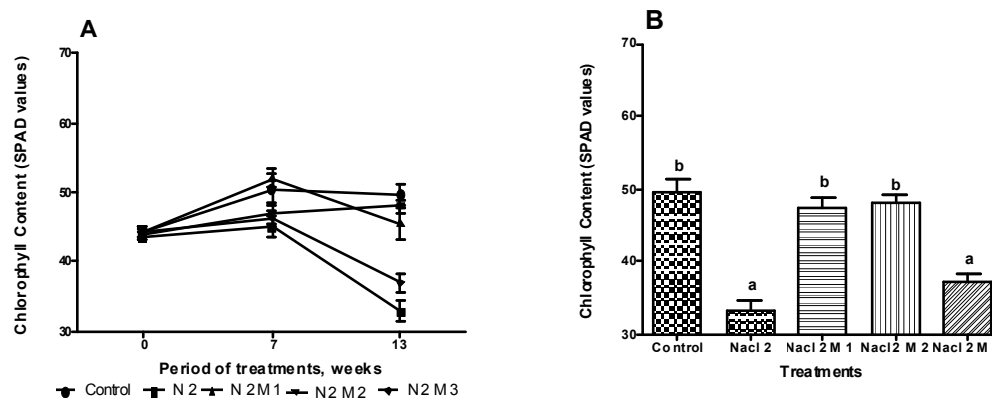


Figure 3.30: Effect of Milsana on Chlorophyll content (SPAD values) of tomato leaves under NaCl stress, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.2.2 Chlorophyll Fluorescence

The chlorophyll fluorescence parameter F_v/F_m , the ratio of variable to maximum fluorescence after dark adaptation, represents the maximum quantum yield of PSII. The F_v/F_m of 0.5%, 1.0% and 2.0% Milsana, treated plants, remained unchanged, compared to control plants (Fig. 3.31).

The combination of treatment with 70 or 140 mM NaCl and 0.5, 1.0% or 2.0% Milsana led to a decrease in the F_v/F_m values of the treated plants, compared to the control plants and were similar to all NaCl treated plants (Fig. 3.32, 3.33). On the contrary, all NaCl treatments with or without Milsana, significantly decreased Chlorophyll Fluorescence. These results agree with those of the growth chamber experiment

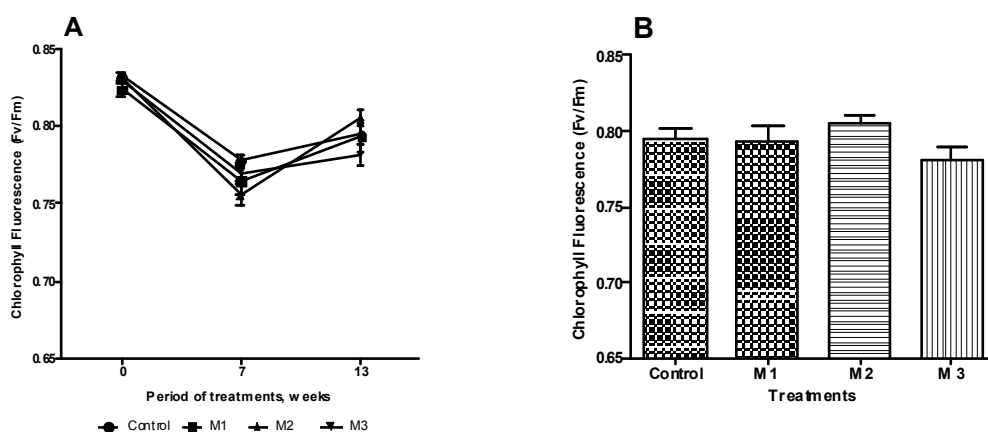


Figure 3.31: Effect of Milsana on Chlorophyll Fluorescence (F_v/F_m) of tomato leaves, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

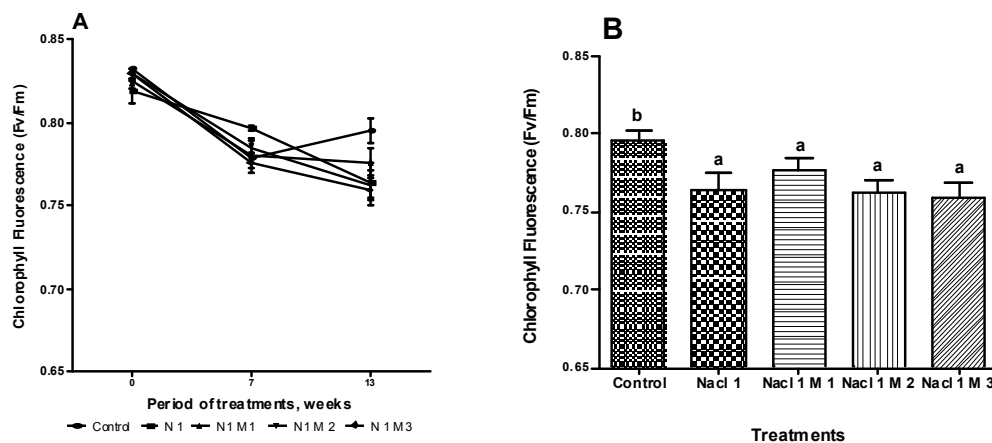


Figure 3.32: Effect of Milsana on Chlorophyll Fluorescence (Fv/Fm) of tomato leaves under NaCl stress, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

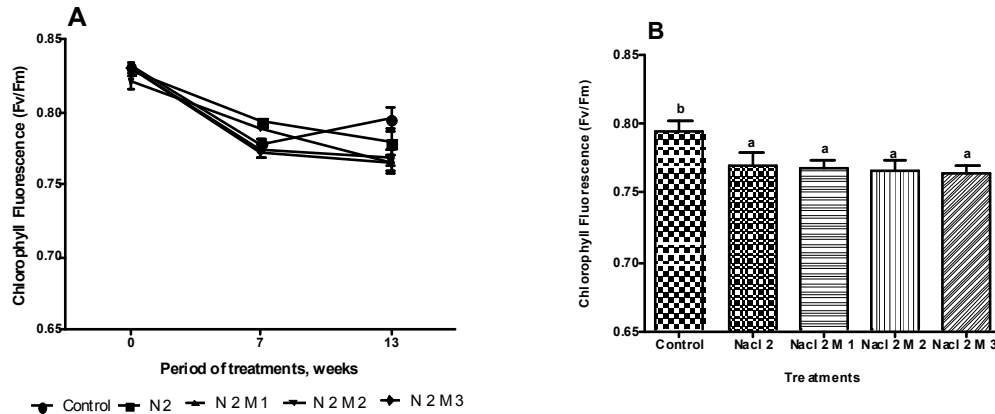


Figure 3.33: Effect of Milsana on Chlorophyll Fluorescence (Fv/Fm) of tomato leaves under NaCl stress, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.2.3 Photosynthesis rate

Leaf photosynthetic rates of 0.5, 1.0, 2.0 % Milsana treated plants were higher to the rates determined for control plants (Fig. 3.34). The lowest rates were observed in the 70 and 140mM NaCl treated plants. The photosynthetic rates of the combined 70mM NaCl + 1.0% or 2.0% Milsana treatments were higher compared to the ones determined for 70mM treated plants (Fig. 3.35). Interestingly all of the combined treatments with 140mM NaCl and Milsana had higher leaf photosynthetic rates compared to 140mM NaCl treated plants at the final week of the experiment (Fig. 3.36). In conclusion, the data indicate that NaCl salinity influences tomato growth through reduction in photosynthetic performance. Net photosynthesis rate generally decreased with NaCl treatments.

Overall Milsana had a positive effect on photosynthesis (Figure 3.34) and exerted a protective effect against NaCl induced reduction in photosynthesis (Figures 3.35, 3.36). These results agree with those of the growth chamber experiment.

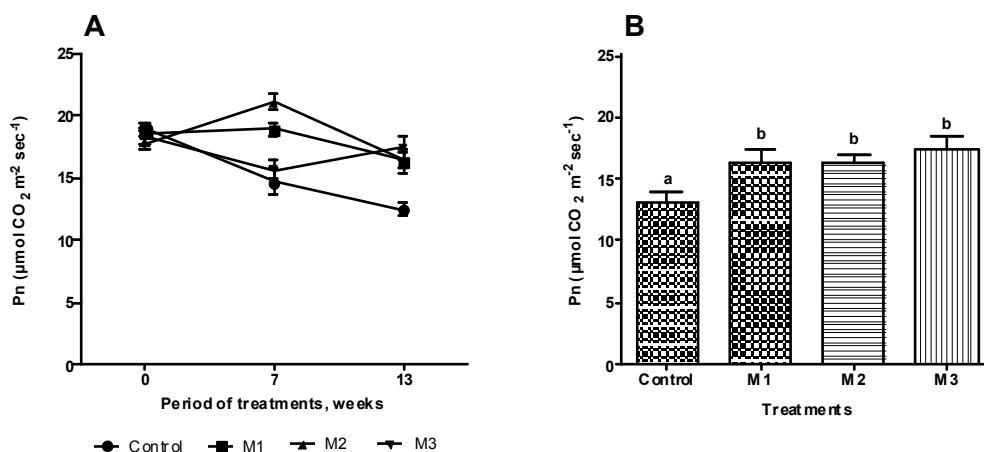


Figure 3.34: Effect of Milsana on Photosynthesis rate (Pn) (μmol CO₂ m⁻² sec⁻¹) of tomato leaves, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week. Values are means ± standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

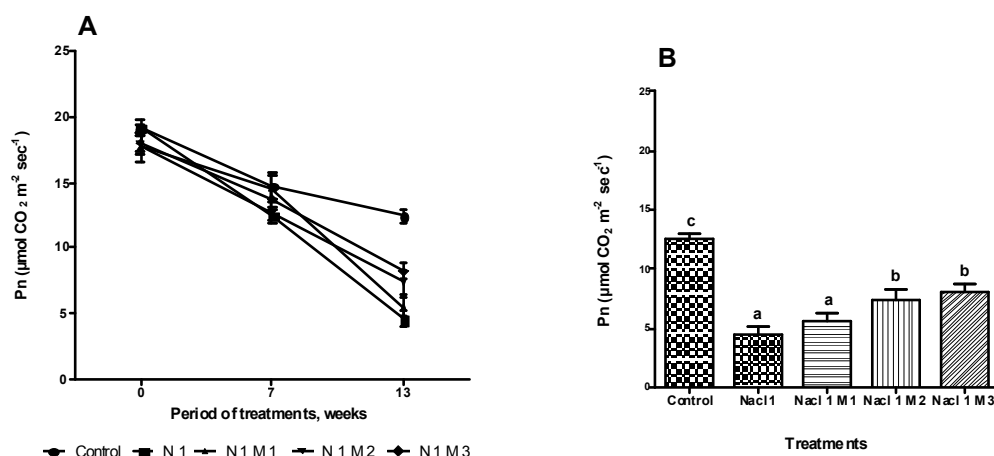


Figure 3.35: Effect of Milsana on Photosynthesis rate (Pn) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}$) of tomato leaves under NaCl stress, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

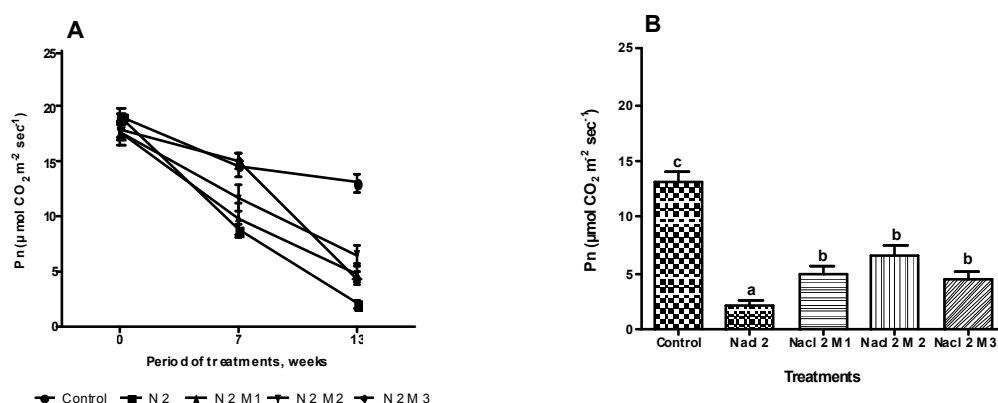


Figure 3.36: Effect of Milsana on Photosynthesis rate (Pn) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}$) of tomato leaves under NaCl stress, A) for the duration of the treatments (13 weeks) B) during the final week (13th week). Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week. Values are means $\pm$ standard error (SE) of 10 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.3 Effect of Milsana Treatments on the Antioxidative Machinery under NaCl Stress

3.2.3.1 Proline content

After the 7th and the 13th week of the experiment, leaf tissue samples were harvested, from the upper (the youngest fully expanded leaf near the tip) and lower (3rd-4th leaves from the base) zones of the plants and used for further analysis. Treatments with Milsana did not lead to any significant increase compared with the control plants in proline content (Fig. 3.37). Treatments with NaCl and combined treatments with NaCl and Milsana had the highest contents of proline compared to the untreated plants (Fig. 3.38, 3.39).

Overall, salinity increased the proline contents in tomato leaves. In general amino acids, such as proline, are related in their metabolic pathways and affected by alteration in enzymatic levels, usually associated with salinity. Our experiment showed that proline accumulation increased with increase in salt stress. These results agree with those of the growth chamber experiment.

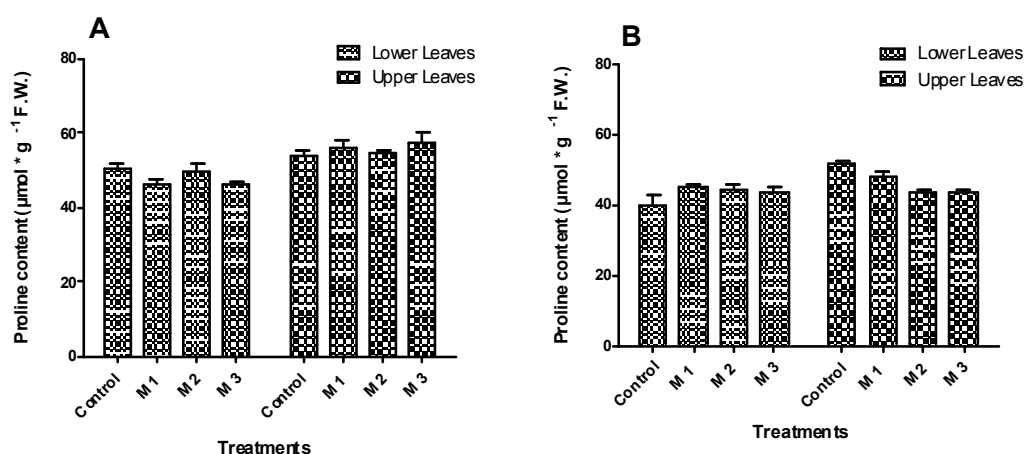


Figure 3.37: Effect of Milsana on Proline content of tomato lower and upper leaves, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions.

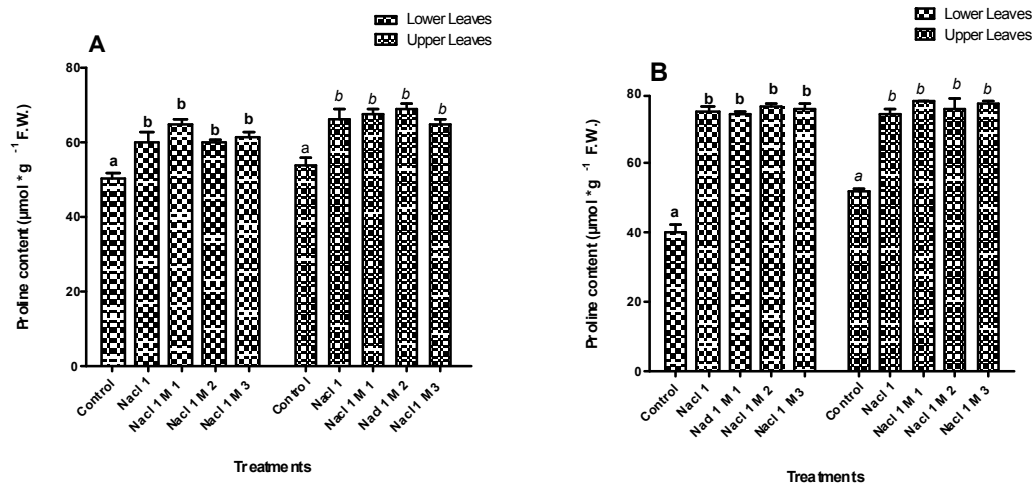


Figure 3.38: Effect of Milsana on Proline content of tomato lower and upper leaves under NaCl stress, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

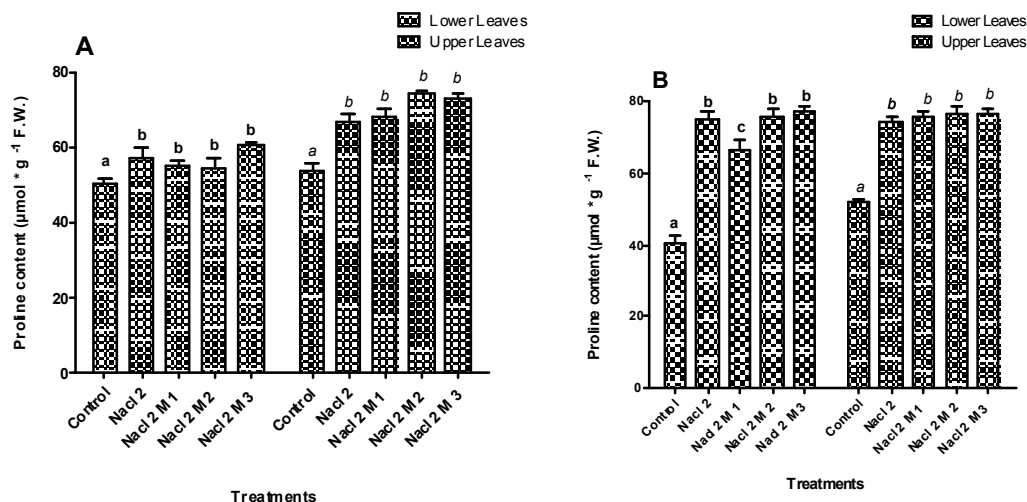


Figure 3.39: Effect of Milsana on Proline content of tomato lower and upper leaves under NaCl stress, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.3.2 Malondialdehyde (MDA) equivalents

Treatments with Milsana did not lead to any significant increase, at the 7th or 13th week, in the lower or upper leaves, compared with the control plants for MDA equivalents (Fig. 3.40). In lower leaves at the 7th week all MDA equivalents are similar. In the upper leaves at the 7th week, the combined treatment of 70mM NaCl + 0.5, 1.0% or 2.0% Milsana exhibited a similar MDA equivalent compared to untreated plants, and the 70mM NaCl treated plants showed a lower MDA equivalent compared to all treated and untreated plants. It is also worth mentioning that the MDA equivalent, at the 13th week on lower leaves, of 70mM NaCl treated plants was lower compared to the rates determined for the combination of treatments of 70mM NaCl + 1% or 2.0% Milsana and untreated plants (Fig. 3.41). The combined treatment of 140mM NaCl + Milsana exhibited a lower MDA equivalent compared to 140mM NaCl treated plants. The MDA equivalent of 140mM NaCl treated plants was higher compared to the rates determined for all treated and untreated plants on lower and upper leaves at the 7th week (Fig. 3.42). In upper and lower leaves at the end of the experiment (13th week), the MDA equivalent of the untreated plants and combined plants with 140mM NaCl and 0.5% or 2.0% Milsana treatments had a lower MDA equivalent compared to 140mM treatments.

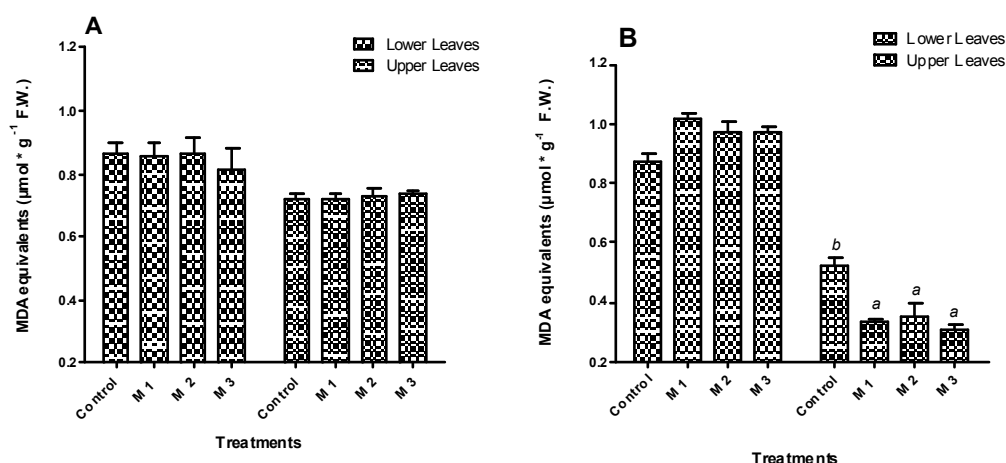


Figure 3.40: Effect of Milsana on Malondialdehyde (MDA) equivalents of tomato lower and upper leaves, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

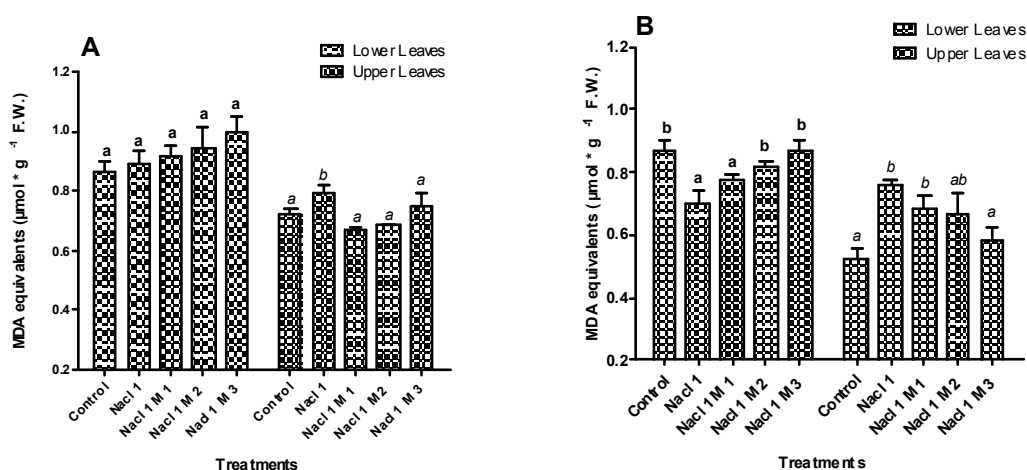


Figure 3.41: Effect of Milsana on Malondialdehyde (MDA) equivalents ($\mu\text{mol} \cdot \text{g}^{-1} \text{ F.W.}$) of tomato lower and upper leaves under NaCl stress, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

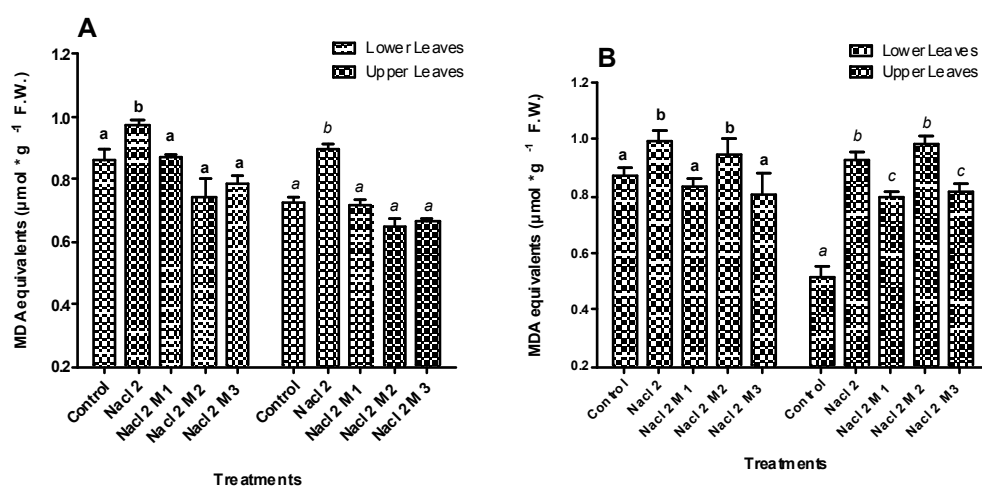


Figure 3.42: Effect of Milsana on Malondialdehyde (MDA) equivalents of tomato lower and upper leaves under NaCl stress, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.3.3 Trolox equivalents Antioxidant capacity

Antioxidant activities were determined by TEAC (Trolox equivalent antioxidant capacity). Treatments with Milsana did not lead to any significant increase in Trolox equivalents antioxidant capacity compared the control plants (Fig. 3.43). The combined treatment of 70mM NaCl + 2.0%Milsana exhibited a higher Trolox equivalent compared to 70mM NaCl treated plants on lower and upper leaves at the 7th week (Fig. 3.47). At the end of the experiment (13th week) in upper leaves the combination of treatments with 70mM NaCl and 0.5% or 2.0% Milsana treated plants had a higher Trolox equivalents (Fig. 3.44). The combined treatment of 140mM NaCl + 2.0% Milsana and untreated plants led to a decrease of Trolox equivalent compared to other treated plants. Treatments with 140mM NaCl had the highest Trolox equivalents compared to all treated and untreated plants in lower and upper leaves at the 13th week (Fig. 3.45). These results agree with those of the growth chamber experiment.

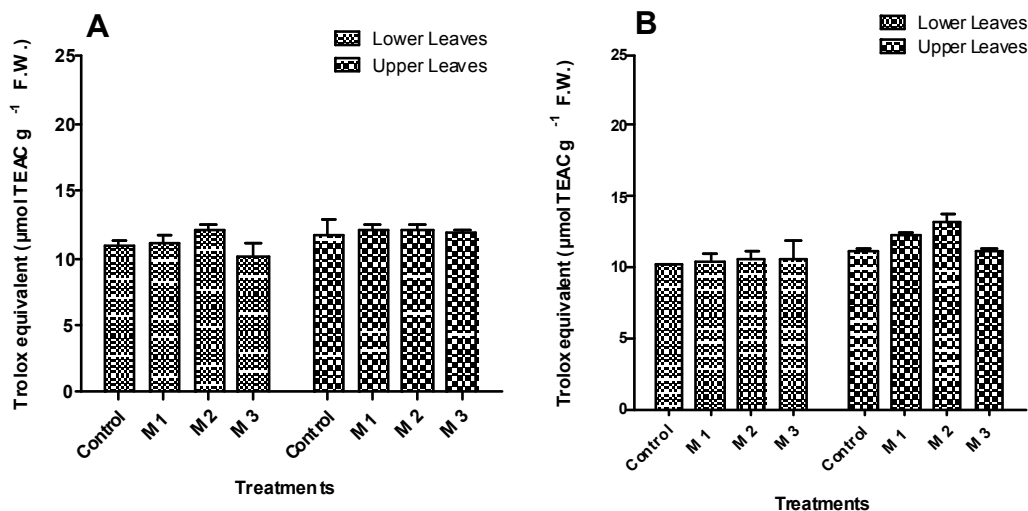


Figure 3.43: Effect of Milsana on Trolox equivalents Antioxidant capacity of tomato lower and upper leaves, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

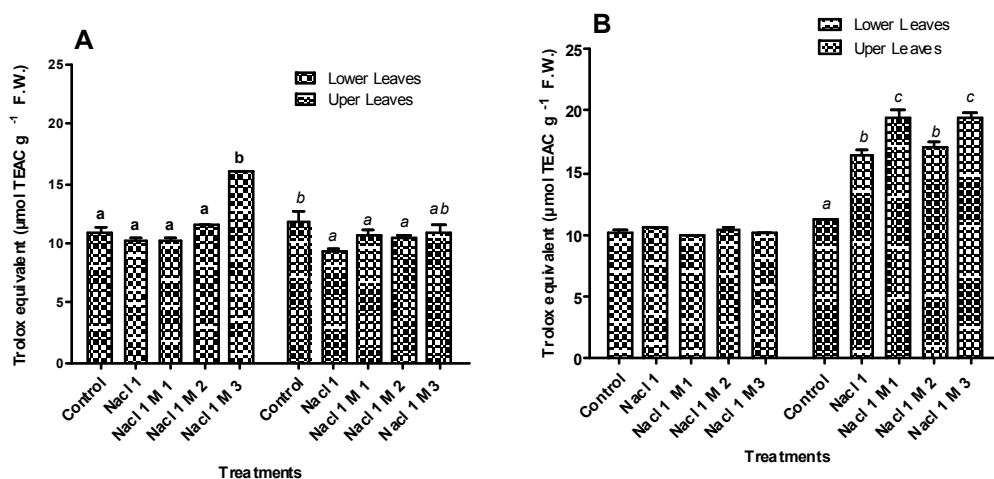


Figure 3.44: Effect of Milsana on Trolox equivalents Antioxidant capacity of tomato lower and upper leaves under NaCl stress, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

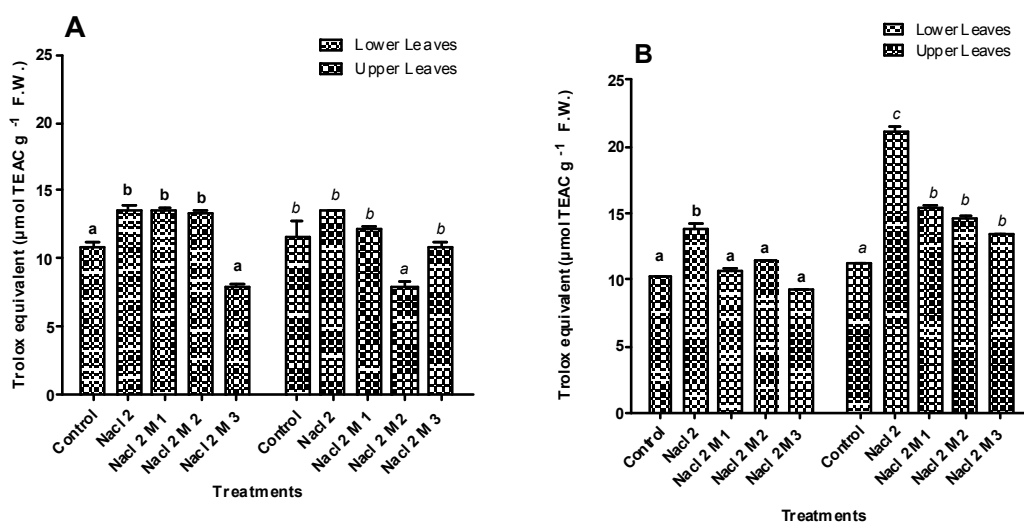


Figure 3.45: Effect of Milsana on Trolox equivalents Antioxidant capacity of tomato lower and upper leaves under NaCl stress, A) at the 7th week, B) at the 13th week of the experiment. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 3 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, P=0.05).

3.2.4 Effect of Milsana Treatments on Fruit Quality of Tomato Plants under NaCl Stress

3.2.4.1 Fruit colour measurements

Colour of tomato fruits is used to determine ripeness as the fruits change colour during the process. On harvest day, 10 fruit of each treatment were used to measure chromaticity values. Measurements of the chromaticity values (L^* , C^* , h^*) were taken on whole fruit and puree of tomato. Fruit colour and puree of tomato was measured using a Konica Minolta CR 400 Colorimeter (Konica Minolta), expressing colour change. The colour variation was measured directly on the fruit. The colour was recorded using L^* (lightness), C^* (chroma), and h^* (hue angle) values. Measurements of the chromaticity values (L^* , C^* , h^*) in the fruit and puree of tomato did not statistically differ either in the absence or in the presence of the NaCl or Milsana treatments (Table 3.2).

Table 3.2: Effect of Milsana treatments on fruit and puree colour parameters (L^* (lightness), C^* (chroma), and h^* (hue angle) values) of tomato plants under NaCl stress. Values are means $\pm$ standard error (SE) of 5 repetitions.

Treatments	Fruit colour parameters (Chromaticity values)			Puree colour parameters (Chromaticity values)		
	L^*	C^*	h^*	L^*	C^*	h^*
Control	42.84 $\pm$ 1.49	45.26 $\pm$ 1.45	46.62 $\pm$ 2.09	29.56	11.88	83.03
M1	41.72 $\pm$ 0.64	44.81 $\pm$ 1.28	42.92 $\pm$ 0.95	27.82	10.86	84.10
M2	41.94 $\pm$ 1.14	42.88 $\pm$ 1.59	43.59 $\pm$ 1.41	29.50	10.95	83.45
M3	43.58 $\pm$ 1.04	44.66 $\pm$ 1.07	42.74 $\pm$ 2.03	28.63	11.02	84.22
NaCl 1	40.80 $\pm$ 1.46	43.79 $\pm$ 2.15	42.49 $\pm$ 2.73	30.88	11.59	85.35
NaCl 1 M1	42.83 $\pm$ 0.92	48.12 $\pm$ 0.78	42.88 $\pm$ 1.73	30.48	11.41	84.77
NaCl 1 M2	42.46 $\pm$ 1.12	47.57 $\pm$ 1.43	42.41 $\pm$ 1.81	28.99	10.82	83.94
NaCl 1 M3	43.85 $\pm$ 1.44	50.48 $\pm$ 1.13	45.44 $\pm$ 1.68	29.16	10.88	83.55
NaCl 2	41.61 $\pm$ 1.68	46.44 $\pm$ 1.72	42.43 $\pm$ 2.83	29.53	10.89	83.49
NaCl 2 M1	41.78 $\pm$ 0.98	47.43 $\pm$ 1.29	41.16 $\pm$ 1.70	28.96	10.87	82.50
NaCl 2 M2	42.91 $\pm$ 1.44	45.41 $\pm$ 1.49	42.12 $\pm$ 2.23	28.74	10.65	81.74
NaCl 2 M3	42.99 $\pm$ 0.98	48.05 $\pm$ 1.00	42.98 $\pm$ 2.18	28.72	10.61	81.33

3.2.4.2 Effect of Milsana on Quality of Tomato Fruits

At the end of the experiment tomato fruits from plants were harvested and used for further analysis. The exposure of plants to salinity resulted in a significantly high number of fruits per plant suffering from blossom-end rot (Fig. 3.46, 3.47). Treatments with Milsana exerted a significant effect on the occurrence of BER (Table 3.3). Fruits from plants treated with the combination of NaCl + Milsana did not suffer from blossom-end rot.

Salinity is a complex environmental constraint that presents two main components: an osmotic component due to decrease in the external osmotic potential of the soil solution and an ionic component linked to the accumulation of ions which become toxic at high concentrations (mainly Na⁺ and Cl⁻). The accumulation of these elements leads to a decrease of the absorption availability of essential elements (e.g. potassium and calcium) (El-Bassiouny et al., 2004).

Table 3.3 Effects of Milsana on the incidence of blossom-end rot of tomato fruits under NaCl stress.

Treatments	Number of fruits per treatment	%Fruits with BER per treatment
Control	110	0
M1	90	0
M2	105	0
M3	101	0
NaCl 1	106	58
NaCl 1 M1	97	0
NaCl 1 M2	103	0
NaCl 1 M3	97	0
NaCl 2	99	78
NaCl 2 M1	89	12
NaCl 2 M2	85	12
NaCl 2 M3	88	19

Tomato plants grown under salinity stress often develop blossom-end rot (BER) (Fig. 3.49), therefore the fruits produced are unsuitable for sale (unmarketable) (Cuartero and Fernández-Muñoz, 1998).

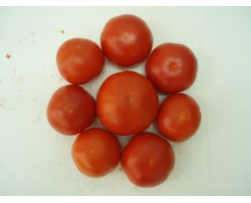


Figure 3.46: Fruit from tomato plants treated with 140mM NaCl

Blossom end rot is a physiological disorder that occurs wherever tomatoes (and peppers) are grown. It is a non-infectious disease caused by low levels of calcium in tomato fruit. BER occurs frequently where large fluctuations in soil moisture are allowed to occur. Rapid plant growth, low potassium and calcium, excess magnesium and nitrogen, excessive salinity, root damage, and high relative humidity all predispose plants to Blossom-end Rot.

Cultivars differ in their susceptibility to blossom-end rot. Increasing salinity leads to higher occurrence of blossom end rot in tomato fruits and is also known to have negative effects in water uptake and plant growth (Albahou and Green, 1997). The deficiency of Ca^{2+} has a negative effect on growth of tomato fruits. One of the consequences of this deficiency/problem is the susceptibility of tomato fruits to blossom-end rot. Increase of salinity results in a linear decrease of the rate of Ca^{2+} uptake (Adams and Ho, 1993).

A.

TREATMENTS			
Control	0.5% Milsana v/v	1.0% Milsana v/v	2.0% Milsana v/v
			

B.

TREATMENTS				
Control	70mM NaCl	70mMNaCl+0.5 % Milsana v/v	70mMNaCl+1.0% Milsana v/v	70mMNaCl+2.0% Milsana v/v
				

C.

TREATMENTS				
Control	140mM NaCl	140mMNaCl+0.5% Milsana v/v	140mMNaCl+1.0 % Milsana v/v	140mMNaCl+2.0 % Milsana v/v
				

Figure 3.47: Effect of Milsana on quality of tomato fruits A) Plants were treated with 0.0, 0.5, 1.0 or 2.0 % v/v Milsana by spraying once a week for 13 weeks. B) Plants were treated with 70 mM NaCl, 70 mM NaCl + 0.5% Milsana, 70 mM NaCl + 1.0% Milsana and 70 mM NaCl + 2.0% Milsana once a week for 13 weeks. C) Plants were treated with 140 mM NaCl, 140 mM NaCl + 0.5% Milsana, 140 mM NaCl + 1.0% Milsana and 140 mM NaCl + 2.0% Milsana once a week for 13 weeks.

3.2.4.3 Effect of Milsana Treatments on °BRIX (Total Soluble Solids) in Tomato Juice Samples under NaCl Stress

Measurements of the °BRIX in the puree of tomato did not statistically differ among treatments either in the absence or in the presence of the NaCl or Milsana treatments.

3.2.4.4 Effect of Milsana Treatments on Number and Weight of Tomato Fruit under NaCl Stress

Total fruit number and weight of harvested tomatoes were determined at the end of the experiment. Results indicated that both treated (NaCl and / or Milsana) and untreated plants produced the similar fruit number. Tomato fruit weight per treatment was reduced by increasing salinity. Milsana did not affect the total number and weight of tomato fruit under NaCl stress (Table 3.4).

Table 3.4: Effect of Milsana treatments on number and weight of tomato fruit under NaCl Stress

Treatments	Total number of fruits	Total weight of fruits
Control	110	5560
M1	90	4382
M2	105	4508
M3	101	5186
NaCl 1	106	4626
NaCl 1 M1	97	4182
NaCl 1 M2	103	3928
NaCl 1 M3	97	4440
NaCl 2	99	3924
NaCl 2 M1	89	3594
NaCl 2 M2	85	3660
NaCl 2 M3	88	3784

3.2.4.5 Effect of Milsana Treatments on Firmness-Consistency of Tomato Fruit under NaCl Stress

Firmness of each tomato was assessed as the force needed to puncture the skin at two different, opposite halves in an equatorial area on each tomato using a penetrometer. Tomato fruit firmness (Kg) of 0.5, 1.0, 2.0 % Milsana treated plants were similar to the rates determined for control plants (Fig. 3.48). The lowest rates were observed in the 70 and 140mM NaCl treated plants (Fig. 3.49, 3.50). Interestingly the fruit firmness of the combined 70mM NaCl + 1.0% or 2.0% Milsana treatments were higher compared to 70mM NaCl treated plants (Fig. 3.49). Finally the combined treatment of 140mM NaCl + 0.5 or 1.0% or 2.0% Milsana exhibited a higher firmness rate compared to 140mM NaCl treated plants (Fig. 3.50).

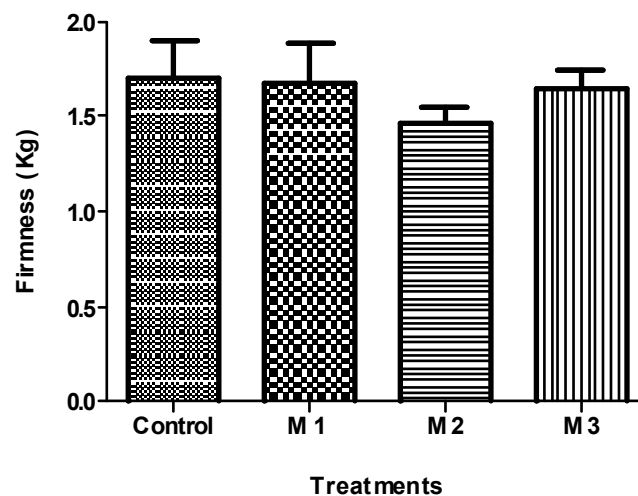


Figure 3.48: Effect of Milsana on firmness of tomato fruits. Plants were treated with 0.0 (Control), 0.5 (M1), 1.0 (M2) or 2.0 % v/v (M3) Milsana by spraying once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 5 repetitions.

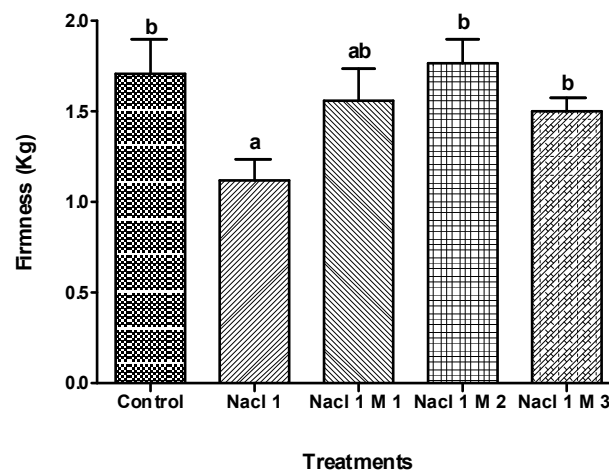


Figure 3.49: Effect of Milsana on firmness of tomato fruits under NaCl stress. Plants were treated with 70 mM NaCl (NaCl1), 70 mM NaCl + 0.5% Milsana (NaCl1-M1), 70 mM NaCl + 1.0% Milsana (NaCl1-M2) and 70 mM NaCl + 2.0% Milsana (NaCl1-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 5 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

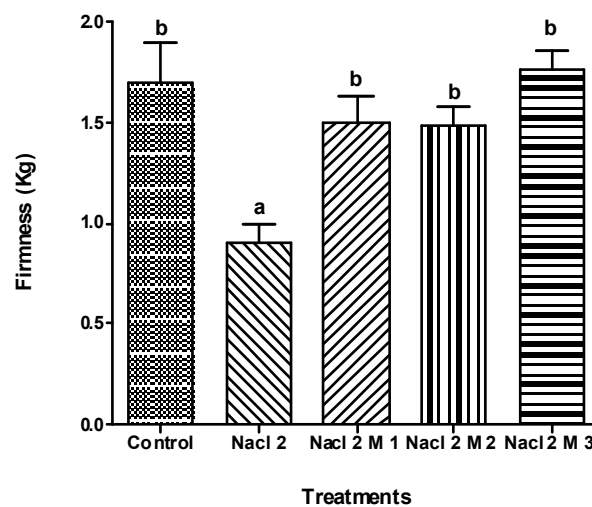


Figure 3.50: Effect of Milsana on firmness of tomato fruits under NaCl stress. Plants were treated with 140 mM NaCl (NaCl2), 140 mM NaCl + 0.5% Milsana (NaCl2-M1), 140 mM NaCl + 1.0% Milsana (NaCl2-M2) and 140 mM NaCl + 2.0% Milsana (NaCl2-M3) once a week for 13 weeks. Values are means $\pm$ standard error (SE) of 5 repetitions. Values which differ significantly from one another are marked with a different letter (Duncan, $P=0.05$).

3.2.4.6 Overall Result Overview

The results of this study suggest a double role for Milsana in tomato plants. Milsana spraying increased plant height, chlorophyll content and photosynthetic rates, suggesting a positive role in plant growth. Furthermore when Milsana was sprayed in plants treated with NaCl it showed a protective role over salinity.

More specifically chlorophyll content and photosynthetic rates were increased in these combined Milsana +NaCl treatments, as compared to NaCl only treatments (without Milsana). In addition values for antioxidant parameters such as antioxidant capacity and lipid Peroxidation in Milsana +NaCl treated plants were similar to the ones determined for control plants, whereas NaCl only treated plants presented significant perturbation in antioxidant parameters.

Finally, the two concentrations of NaCl variably affected plant growth and function. Both concentrations negatively affected photosynthesis and chlorophyll content, whereas they did not affect fluorescence and proline content. Mild salt stress (70mM) exerted a negligible effect on the antioxidative capacity of the treated plants, in contrast to higher concentration of NaCl (140mM), which increased ROS generation (higher MDA and Trolox equivalent).

CHAPTER 4

Conclusions

4.1 Conclusions

In this study, the effect of Milsana (at three application levels) on tomato plants under physiological conditions and under NaCl stress was examined. Milsana is a formulated plant extract from *Reynoutria sachalinensis*.

The effect of the Milsana was tested in two different sets of experiments, one carried out in a growth chamber under controlled conditions and the other carried out in a greenhouse. In both growth conditions, almost all the parameters examined were significantly affected and the main conclusions are:

- Milsana application to control plants positively affected plant growth and physiology:
 - Enhanced plant growth, evident by the observed increase in height.
 - Milsana treatments partially raised photosynthetic activity, in terms of chlorophyll content and photosynthesis rate.
 - Milsana did not exert any excess stress on the plant evident by the lack of any measurable antioxidative response, compared to untreated plants.
- NaCl application negatively affected almost all the parameters examined, especially at high concentration:
 - Reduced plant growth and toxicity symptoms were observed.
 - Photosynthesis breakdown was in direct relation with the reduction in chlorophyll content and photosynthesis rate.
 - Induced activation of the antioxidative mechanism suggesting salinity induced oxidative stress, further supported by the increase in lipid peroxidation products (MDA).
- Combined treatments of Milsana + NaCl were shown to reverse some - negative effects of salinity stress,
 - Reduction in NaCl – induced stress symptoms was observed.

- Levels of most parameters examined were reverted at physiological values. Especially:
 - photosynthetic activity
 - antioxidative mechanism
- Interestingly the quality of fruit of the combined NaCl + Milsana treatments was higher compared to NaCl treated plants in terms of:
 - Increase of firmness (Kg)
 - Significant reduction of salinity induced blossom end rot (BER)

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