

<sup>1</sup>Hungarian Department of Biology and Ecology, Babes-Bolyai University, Cluj-Napoca, Romania  
<sup>2</sup>Department of Horticulture, Sapientia Hungarian University of Transylvania, Targu Mures, Romania

## Enhancement of biomass production, salinity tolerance and nutraceutical content of spinach (*Spinacia oleracea* L.) with the cuticular wax constituent triacontanol

Bernat Tompa<sup>1</sup>, Janos Balint<sup>2</sup>, Laszlo Fodorpataki<sup>1,2\*</sup>

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### Summary

The present study investigates the effect of foliar application of triacontanol (TRIA) on various physiological parameters with regard to crop yield and quality attributes of spinach (*Spinacia oleracea* L.) under normal growth conditions and salinity stress. Plantlets were grown for 21 days in perlite-containing pots supplemented with Hoagland's nutrient solution, then they were subjected to 0 (control) or 150 mM NaCl. Two concentrations of TRIA (25 nM and 1  $\mu$ M) were applied during seed germination and as foliar spray treatment, in itself or simultaneously with salt stress at 4-day intervals for three weeks. Exogenous application of TRIA enhanced germination energy and capacity, as well as shoot and root biomass of young spinach plants. Inhibition of net CO<sub>2</sub> assimilation (Pn) and of potential quantum yield of photosystem II (Fv/Fm), caused by salt stress, was significantly reduced by treatment with triacontanol. Increment of carotenoid pigment and ascorbate (vitamin C) content, as well as reduction of membrane lipid peroxidation due to triacontanol treatment improves the health-promoting quality of spinach leaves developed under high salinity conditions. The presented results offer a novel solution for optimization of spinach cultivation on soils affected by high salinity, as well as for an increased content of health-promoting metabolites of spinach leaves upon human consumption.

### Introduction

Plant growth regulators (PGRs) are widely used in modern agriculture and horticulture. Approximately 40 active ingredients are in use and appropriate combination/concentration of these may significantly improve the overall plant growth, yield and quality (RADEMACHER, 2015). PGRs include naturally occurring plant growth substances (translocatable phytohormones or local bioregulators) and synthetic compounds, which mostly are chemical analogs that alter hormone levels (hormone releasing agents or synthesis inhibitors) or hormone sensitivity (HAJAM et al., 2017). Exogenous application of PGRs provides an alternative approach to counteract the adverse effects of abiotic stresses including high salinity (KHANAM and MOHAMMAD, 2018), heavy metal toxicity (ASGHER et al., 2015) or drought stress (ULLAH et al., 2012), and increase stress tolerance in plants by regulating gene expression and a number of physiological-biochemical processes (UPRETI and SHARMA, 2016). Conventional plant breeding methods for improving plant tolerance to abiotic stress are time consuming, laborious, costly and dependent on existing genetic variability, while application of PGRs can be a wise strategy to maximize plant productivity under adverse growth conditions. The most suitable and efficacious technique is the foliar application of diluted aqueous solutions of PGRs for optimization of genetic potential of a crop. It is expected that the need to raise agricultural production will lead to the increased use of PGRs.

Out of various PGRs, triacontanol (TRIA) is a natural constituent of plant epicuticular waxes, which can exert its stimulatory effects at considerably low concentrations. Due to its non-toxic and not-polluting properties, TRIA is known to be a potential plant growth stimulator of many agricultural and horticultural crops (NAEEM et al., 2012). The exogenously applied TRIA has been reported to enhance vegetative growth (SARWAR, 2017), water and mineral nutrients uptake (ASADI KARAM et al., 2017a), accumulation and synthesis of compatible organic compounds (SHAHBAZ et al., 2013). TRIA modulates hormonal balance (PANG et al., 2020), nitrogen assimilation (NAEEM et al., 2009) and activities of antioxidant enzymes (ASADI KARAM et al., 2017b), leading to the enhancement of growth and quality characteristics of crops. Owing to the high-efficiency and broad-spectrum of TRIA, it plays an important role in plant growth and development, but mechanisms by which TRIA exerts its effects remain unelucidated. Furthermore, TRIA may play a crucial role in improvement the plant tolerance against several abiotic stresses such as transplantation shock, heavy metal toxicity or drought, and it can help ameliorate the harmful effects of different adverse growth conditions (ZAID et al., 2020).

Salt stress in one of the major abiotic factors limiting production and quality of food crops worldwide. Approximately 20% of total cropland and 33% of irrigated agricultural land are affected by salinity. It is expected that by 2050, half of the croplands worldwide will become salinized and world population will surpass 9.5 billion people (URI, 2018). Consequently, the world population will not have sufficient fertile soils to grow the food it requires, thus there is a strong need for more productive and stress-tolerant crops in the globally shrinking agricultural lands. Soil salinization is accelerating as a result of intrusion of water contaminated with salt ions (poor drainage), excess irrigation and fertilization practices, soil erosion (deforestation) on large scale, and slow natural salt leaching processes (MOREIRA BARRADAS et al., 2015; SINGH, 2015; CUEVAS et al., 2019).

Salt stress manifests in physiological drought, which reduces turgor pressure leading to reduction in the cell expansion and growth. High salinity is known to inhibit plant growth mainly due to osmotic stress in a first phase (RASOOL et al., 2013), toxicity of sodium and chlorine ions on a longer time scale of exposure (HASANUZZAMAN et al., 2013), and hormonal imbalance (FAROOQ et al., 2015). The combination of these factors is responsible for the oxidative stress caused by over-accumulation of reactive oxygen species (ROS). Excess production of the extremely unstable ROS results in disruption of membrane lipids via peroxidation of unsaturated fatty acids, which causes production of highly reactive lipid peroxidation-derived molecules such as 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal, malondialdehyde and acrolein. These unstable molecules are named reactive carbonyl species (RCS) and high concentrations of RCS can cause irreversible damage in plant cells, which ultimately leads to cell death (YALCINKAYA et al., 2019). To scavenge ROS, plants have evolved a highly integrated defense system consisting of several antioxidants, which provide protection from the oxidizing effects of ROS and

\* Corresponding author

counteract their electron acquiring properties. Lipid peroxidation is inhibited by naturally occurring carotenoids ( $\beta$ -carotene, zeaxanthin, lutein), because these photosynthetic pigments perform an essential photoprotective role by quenching triplet state chlorophyll molecules, scavenging singlet oxygen and dissipating of the harmful excess excitation energy under stress conditions. In chloroplasts, peroxisomes and cytosol, ascorbate (vitamin C) has a key role in hydrogen peroxide scavenging via ascorbate peroxidase, while in the apoplast and vacuoles, it is the main reductant of phenolic radicals generated under oxidative stress. Being a major component of the Foyer-Halliwell-Asada cycle, ascorbate helps to modulate oxidative stress tolerance by controlling ROS detoxification, both alone and in cooperation with glutathione (GSH). Besides its antioxidative role, ascorbate has an important role in a complex and well-orchestrated plant response network to environmental stress, performing multiple tasks in redox signaling, regulation of enzyme activities, modulation of stress-related genes expression, biosynthesis of phytohormones and growth regulation (VELJOVIĆ-JOVANOVIĆ et al., 2017).

Like in case of most glycophytes, yield and quality of spinach is adversely affected by salt stress. For example, fresh weight, area and  $K^+$  content of leaves, photosynthetic rate (Pn), ascorbate (AsA) and glutathione (GSH) content have been found to be reduced in spinach under saline conditions (HOQUE et al., 2007; ORS and SUAREZ, 2017). Spinach belongs to the Amaranthaceae family, it is an annual (rarely biennial) plant regarded as the second most nutritious leafy green crop in the world. Nutritionally, spinach is a rich source of vitamin A (9380 IU/100 g of edible portion), vitamin  $K_1$  (483 mg/100 g of edible portion), folate (194 mg/100 g of edible portion), which are essential for various metabolic activities in the human body (USDA, 2019). Spinach is a good source of antioxidants and has one of the highest ORAC (oxygen radical absorbance capacity) values of any vegetable (OU et al., 2002). The health benefits of spinach include anti-cancer and anti-obesity properties, prevention of macromolecular oxidative damage and age-related macular degeneration (ROBERTS and MOREAU, 2016).

The recent development of baby leaf spinach coupled with an upswing of nutrition concerns has been responsible for a considerably increased consumption of spinach in the last decade (MORELOCK and CORRELL, 2008). It is a moderately salt-tolerant plant, which allows its cultivation on croplands where salinity problem already exists or is likely to be salinized in the near future. Thus, there is a strong need for further improvement of new technologies in order to increase salt tolerance of spinach, as well as to manage the present and future challenges of low productivity and increasing global demands. According to the publications available at present, there is no information pertaining to effects of triacontanol on physiological and biochemical parameters of spinach plants under salt stress conditions, except for one of our recent studies (TOMPA and FODORPATAKI, 2021). Therein, we show that triacontanol increases the fresh shoot biomass of young spinach plants and neutralizes the deleterious effects of salt stress on the photosynthetic pigment content of leaves. Our hypothesis is that TRIA acts as a chemical signal that triggers specific reactions in plants, leading to stimulation of physiological processes and enhancement of defense mechanisms under environmental stress conditions. The main objective of this work is to investigate the influence of salt stress, triacontanol and their combination on growth and metabolic processes of spinach, related to productivity, salinity tolerance and health-promoting quality.

### A step towards Sustainable Development Goal 2 “Zero Hunger”

Our study can help improve the use of plant growth regulators and other natural bioactive compounds in cost-effective and environmental-friendly horticultural and agricultural practices, thus enhancing yield and nutritional composition of crop plants under unfavorable

conditions of cultivation, such as high soil salinity related to a dryer and warmer climate. All of these can represent a step toward reaching the Sustainable Development Goal (SDG) #2 “Zero hunger”.

## Materials and methods

### Plant material and growth conditions

The experiments were carried out with spinach (*Spinacia oleracea* L.) plants belonging to the ‘Popey’ cultivar. This was selected from three frequently grown cultivars (‘Popey’, ‘Matador’ and ‘Viroflay’, the seeds being purchased from ZKI and Garafarm Ltd.), largely used in nutrition as fresh leafy vegetables (mainly in salads). Based on previous studies, the ‘Popey’ variety was found to be moderately tolerant to high salinity and was selected for experiments concerning the alleviation of the negative effects of salt stress with triacontanol. For investigation of germination dynamics, uniform sized healthy seeds, 100 in four replications for each treatment setup, were selected and sterilized in 0.1% (w/v) sodium hypochlorite solution for 10 minutes, then washed twice with distilled water. For the other experiments, sterilized seeds were pre-hydrated for 24 hours in aerated distilled water and germinated in polyethylene zipper bags, between filter paper sheets moistened with distilled water. Spinach seeds require cold stratification in order to break the embryonic dormancy cycle and to germinate more uniformly. Therefore, germination temperature in the growth chamber was set at 5.5 °C for a 12-hour dark period and 22 °C for a 12-hour light period, for four days. At the end of the fourth day, the seeds were kept at 22 °C for another three days until the sowing. After the germination period, seedlings with similar size were planted separately in pots filled with perlite, watered regularly with Hoagland’s nutrient solution (pH 6.5) and grown for three weeks in a growth chamber under controlled environmental conditions. The temperature was kept at 22 °C and the relative air humidity at 60%. The daily photoperiod was set to 14 hours of light and 10 hours of darkness, the photon flux density was 260  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  photosynthetically active radiation.

To reveal the physiological effects of TRIA in spinach under normal growth conditions, three experimental variants were set up, each with five plantlets: the control provided with basic Hoagland solution, a series exposed to 25 nM TRIA and another to 1  $\mu\text{M}$  TRIA treatment (triacontanol being purchased from the Nutri-Tech Solutions Pty Ltd). Two concentrations of TRIA were applied as foliar spray in every fourth day for three weeks. Control plants were sprayed with deionized water. To investigate the compensation capacity of TRIA under salt-stressed conditions, four experimental variants were set up, each with five plantlets: the control, a series exposed to 150 mM NaCl, a series treated with 1  $\mu\text{M}$  TRIA and exposed simultaneously to salt stress induced with 150 mM NaCl, and a series treated with 1  $\mu\text{M}$  TRIA, but not subjected to salt stress. NaCl was dissolved in the nutrient solution used for watering the substrate, while TRIA solution was pulverized on the aboveground parts of the plants. Growth conditions during the treatments were similar with those created for the development of seedlings after germination.

### Germination dynamics

Surface sterilized seeds, four repetitions of 100 seeds each, were soaked for 24 hours in different aqueous solutions according to experimental variants: control lots were immersed in distilled water, while treated seeds were covered by aqueous solutions of 1  $\mu\text{M}$  triacontanol or 150 mM sodium chloride, and mixtures of these. After 24 hours, all seeds were put at equal distances from each other in polyethylene zipper bags, between double-layered filter papers thoroughly imbibed with distilled water for control lots and with the appropriate solutions, according to the three different treatments. The seeds were transferred in a growth chamber with 5.5 °C for a 12-hour

dark period and 22 °C for a 12-hour light period, with 70% relative humidity, for 12 days. The filter papers were kept saturated with the treatment solutions. The germinated seeds (with the radicle emerged through the seed tegument) were recorded every third day during the morning hours. Finally, for each treatment, the germination percentage (GP) and the germination energy (GE) were calculated as the average of the four replications.

### Growth and biomass analyses

At 42 days after sowing, plants from each experimental variant were uprooted carefully, were washed with tap water to remove all adhering perlite particles and were dried using blotting papers. Shoot and root lengths were recorded using a measuring scale. The plants were cut to separate roots and shoots, to determine the fresh weights of underground and aboveground vegetative organs. The same plants were then dehydrated in a hot-air oven at 80 °C for three days to determine their dry weights.

### Measurement of leaf gas exchange parameters

Gas exchange parameters were monitored *in situ* on the abaxial side of the fourth fully expanded leaf from the base of stem (developed during the triacontanol treatment), with a Ciras-2 type leaf gas exchange meter (PP Systems). In the measurement chamber the temperature was maintained at 22 °C, CO<sub>2</sub> concentration at 410 μmol mol<sup>-1</sup>, the relative air humidity at 60% and light intensity at 500 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density. Stomatal conductance (Gs), intensity of transpiration (E) and net photosynthetic carbon dioxide assimilation (Pn) were the main physiological parameters registered in the middle of the daily photoperiod.

### Determination of induced chlorophyll fluorescence parameters

Conventional parameters of induced chlorophyll fluorescence were determined *in situ* on the fourth emerging leaf, with an FMS-2 type fluorometer (Hansatech), in order to evaluate energetic efficiency of light use in photochemical reactions of photosystem II (PSII). On dark-adapted leaves, just before the onset of the light period, the ground fluorescence (Fo) was measured by illuminating the leaves with a faint red light flash (0.1 μmol m<sup>-2</sup> s<sup>-1</sup>), while maximum fluorescence (Fm) was recorded during a subsequent saturating light pulse (10000 μmol m<sup>-2</sup> s<sup>-1</sup> for 0.5 s). The potential quantum yield of photosystem II (PSII) was calculated as the Fv/Fm ratio, where Fv is the difference between Fm and Fo (LICHTENTHALER et al., 2005).

### Quantification of photosynthetic pigments

Photosynthetic pigments were extracted from the fourth fully expanded leaf, from the same leaf blade region on which gas exchange and chlorophyll fluorescence measurements were performed *in vivo* straight before pigment determination. Extracts were obtained from 0.25 g of leaves (fresh weight) finely homogenized in 5 mL 80% (v/v) acetone, then the supernatant resulting from centrifugation for 10 min at 4000g and 4 °C was used for absorbance measurements, using a UV-Vis spectrophotometer (Jasco). Concentrations of chlorophyll a, chlorophyll b and carotenoids were calculated according to WELLBURN (1994), by measuring the absorbance of the solution at the wavelengths 663 nm, 646 nm and 470 nm.

### Measurement of malondialdehyde and other TBARS

Oxidative damage of membranes was evaluated on the basis of formation of malondialdehyde (MDA) and other thiobarbituric acid-reactive substances (TBARS) due to peroxidation of unsaturated fatty

acids in membrane lipids. 0.5 g fresh root samples were homogenized with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution in a pre-chilled mortar, the homogenate was centrifuged at 15000 g for 15 min, then 2 mL of the supernatant was transferred in a test tube and 4 mL of 10% (w/v) TCA with 0.5% (w/v) 2-thiobarbituric acid (TBA) were added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. The cooled solution was centrifuged at 10000 g for 5 min, and the absorbance of the supernatant was measured at 532 nm and 600 nm. The absorbance at 600 nm (due mainly to interference of anthocyanin pigments) was deducted from the value obtained at 532 nm. MDA concentration of samples was calculated using the extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> (HEATH and PACKER, 1968).

### Determination of ascorbic acid content and of the reduced to oxidized vitamin C ratio

Ascorbic acid content was determined by homogenizing 0.5 g spinach leaf with 4 ml of 6% trichloroacetic acid in a prechilled mortar. The mixture was centrifuged for 13 min at 15600 g and 4 °C, then 2 ml of supernatant was transferred to sodium phosphate buffer (pH 7.4) containing trichloroacetic acid, dithiothreitol, orthophosphoric acid, ethanolic solution of 2,2'-dipyridyl, N-ethylmaleimide and ferric chloride. After one hour of incubation of this extract mixture at 42 °C with permanent mixing, absorbance of the solution was measured at 525 nm with a spectrophotometer. The oxidized form of vitamin C present in leaf tissues (dehydroascorbate) was reduced to ascorbate by dithiothreitol. Then the total ascorbic acid content was determined photometrically by the 2,2'-dipyridyl method, and concentration of dehydroascorbate was calculated as the difference between total and reduced ascorbate. A standard curve was obtained with known concentrations of pure ascorbic acid dissolved in 5% trichloroacetic acid, in the range of 25-100 nM (KAMPFENKEL et al., 1995).

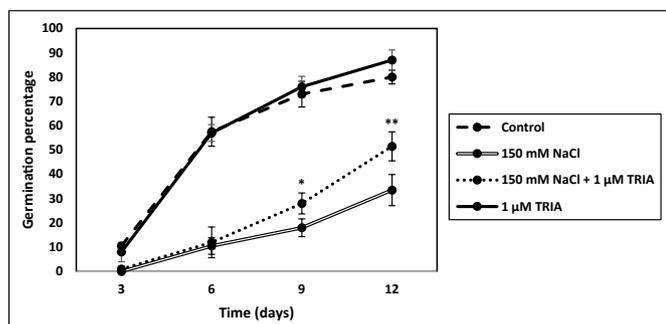
### Statistical analysis

All experimental variants were set in 5 replicates, except for germination, in which case experiments with 100 seeds for each treatment were conducted in four repetitions. Measurements of physiological parameters were repeated three times. Experimental data were statistically processed in R software environment (version 4.1.0.), using the Shapiro-Wilk test for normality and Bartlett's test for homogeneity of variances. Data were represented as the mean ± standard error (SE). The significant differences were determined by the one-way ANOVA test and the post-hoc Tukey HSD test. Differences were considered statistically significant at P < 0.05.

## Results and discussion

### Germination

Germination is an important developmental stage for seedling establishment and therefore it plays a key role in crop production. Seed priming is a simple, low-cost, easily performable technique, which improves the seed performance and provides faster and synchronized germination. Application of 1 μM triacontanol as seed priming substance did not cause spectacular changes in the dynamics of spinach seed germination, but it considerably alleviated the delaying and inhibitory effects of high salinity (Fig. 1). In the case of only TRIA-treated seed lots, the final germination percentage was higher by 8% after twelve days as compared to the control seeds, but this did not represent a major increment, even it was statistically significant. Soil salinity is one of the major constraints of seed germination, because it creates a negative external osmotic potential to prevent water uptake and exerts a toxic effect on the germinating seeds through the excess amount of Na<sup>+</sup> and Cl<sup>-</sup> ions. Ion toxicity and osmotic stress are re-

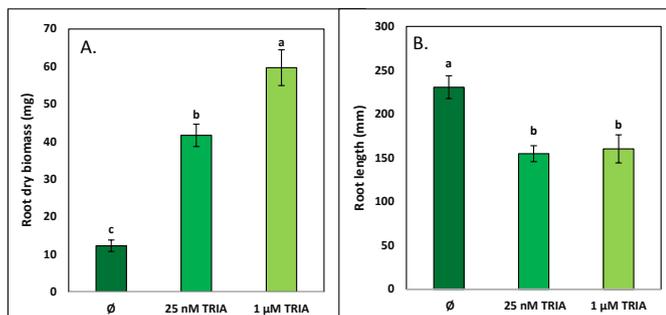


**Fig. 1:** Effects of NaCl and/or triacontanol (TRIA) on germination of spinach seeds. Significant differences between salt-stressed seeds with and without TRIA treatment are marked with \* ( $P < 0.05$ ) and \*\* ( $P < 0.01$ ). Vertical bars represent  $\pm$  standard error from means ( $n=4$ )

responsible for both inhibition and delay of seed germination and seedling establishment. Under a salt stress induced by 150 mM NaCl less than 19% of seeds germinated until the ninth day and around 34% germinated until the end of the 12 days period. These impairments caused by high salinity were significantly counteracted by priming of seeds with 1  $\mu$ M of TRIA: the germination percentage of seeds exposed to 150 mM NaCl was increased from 18% to about 28% on the ninth days, and the final germination percentage was increased from 34% to 52%. A similar conclusion was drawn by SARWAR (2017), when priming with 50  $\mu$ M triacontanol decreased time to start emergence and improved final germination capacity in case of cucumber seeds exposed to salt stress induced by 50 mM NaCl. Seed priming with aqueous solution of 1  $\mu$ M triacontanol helped to maintain a significantly higher germination percentage and reduced the time needed for germination in salt-stressed spinach seeds, thus improving salinity tolerance at this early and sensitive developmental stage. On a longer time scale, better germination greatly contributes to the establishment of vigorous crop stands and an abundant final yield.

### Vegetative growth

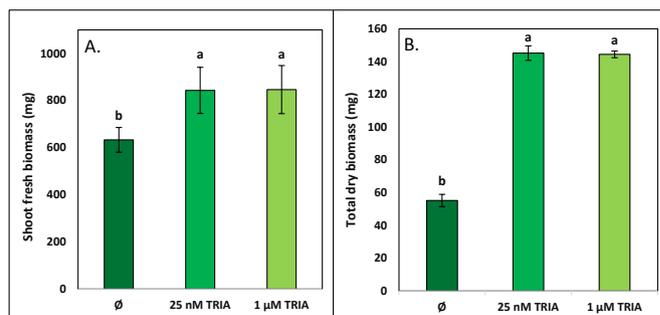
The root system influences plant productivity via its phenotypic traits such as root length, biomass, branch density, volume, and contact area. Foliar application of TRIA at 25 nM and 1  $\mu$ M concentrations appreciably enhanced the root dry biomass (Fig. 2A), but significantly reduced the length of spinach main root axis (Fig. 2B). The highest root dry biomass values were recorded upon treatment with 1  $\mu$ M TRIA. Influence of triacontanol resulted in a shorter, but more densely branched root system, which makes efficient use of a smaller soil volume through the denser network of ramifications. Allocation



**Fig. 2:** Effects of two concentrations (25 nM and 1  $\mu$ M) of triacontanol (TRIA) on the root dry biomass (A.) and root length (B.) of spinach (Ø stands for the control group). Vertical bars represent  $\pm$  standard error from means ( $n=5$ ), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

of assimilates is supposedly redirected to formation of more lateral roots, which increases overall absorption surface for water and mineral nutrients. On the other hand, shorter roots could be detrimental in situations when the soil surface dries out regularly, while longer roots can reach deeper into the soil for moisture. Benefits of a densely branched and shorter root system highly depend on irrigation regimes and cultivation practices. TANTOS et al. (2001) reported a significant increase in the number of roots per plant as result of TRIA application in the rooting media used for the micropropagation of apple and sour cherry.

Because spinach is a leafy vegetable, the fresh biomass of the vegetative shoot is particularly important for its production. Treatment with 25 nM triacontanol of non-stressed plantlets resulted in a considerably increased shoot fresh biomass, while higher TRIA concentration did not lead to higher shoot weight than induced by 25 nM (Fig. 3A). A similar positive influence of triacontanol was observed on total (root and shoot) dry biomass of six weeks old spinach plants. In this case, the stimulatory effect of TRIA principally refers to production of organic metabolites (photosynthates), as water content of plant organs is not modified significantly by triacontanol (Fig. 3B). NAEEM et al. (2011) reported that foliar application of TRIA at 1  $\mu$ M concentration significantly enhanced the length of shoots, the number and surface area of leaves, and the fresh and dry biomass of the wild mint. Increment exerted by TRIA on the yield and content of active constituents (menthol, L-methone, isomenthone and menthyl acetate) of the essential oil augmented the medicinal value of this herb. Triacontanol did not stimulate the growth of vegetative organs in proportion to its concentration, which may suggest that it has only a signaling, triggering role in this process and that its effect is amplified during signal transduction. Cascading effect results in enhanced metabolism processes and the accumulation of various intermediate compounds, for example elevated intracellular  $Ca^{2+}$  levels leading to changes in gene expression (ISLAM et al., 2021). Treatment with very small concentrations of TRIA improves the vegetative growth and biomass production of young spinach plants in a cost-effective way, which is not negligible given the growing demands for baby spinach.

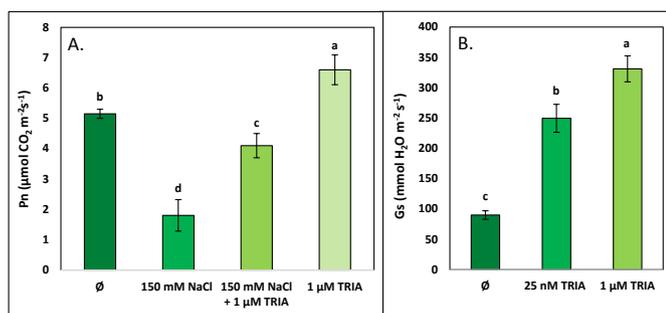


**Fig. 3:** Effects of two concentrations (25 nM and 1  $\mu$ M) of triacontanol (TRIA) on fresh shoot weight (A.) and total dry biomass (B.) of young spinach plants (Ø stands for the control group). Vertical bars represent  $\pm$  standard error from means ( $n=5$ ), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

### Photosynthetic performance

Photosynthesis is one of the most fundamental and intricate physiological processes in all green plants, which is determining crop production, but it is severely affected by several unfavorable environmental conditions, including soil salinity. A simultaneous measurement of the responses of leaf gas exchange and induced chlorophyll fluorescence to salt stress provides an effective mean to understand the main limitations of photosynthesis *in vivo*. Salt stress alters photosynthetic mechanisms by disrupting thylakoid membranes,

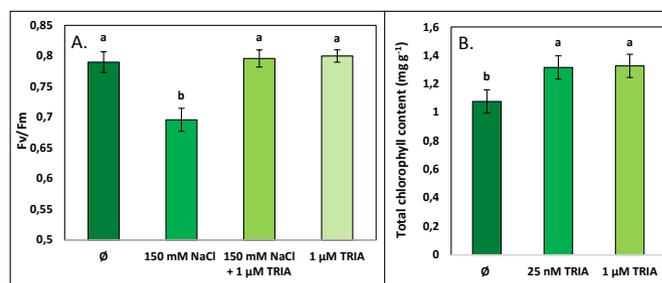
modifying the electron transport chain, changing enzymatic activity during chlorophyll synthesis, and the coordinated functioning of the different steps of the Calvin cycle. Net photosynthetic rate (Pn), measured through carbon dioxide uptake of spinach leaves, was drastically decreased (by an average of 65%) in plants exposed to 150 mM NaCl (Fig. 4A). This reduction was alleviated significantly by simultaneous application of 1  $\mu$ M triacontanol, while in case of non-stressed plants TRIA caused a 28% increment of net carbon dioxide assimilation rate in comparison to control plants. TRIA stimulated the opening of stomata proportionately to its quantity used, thus enhancing their conductivity for carbon dioxide and for water vapor (Fig. 4B). Similar results were reported by SHAHBAZ et al. (2013) for canola seedlings, as well as by NAEEM et al. (2011) for wild mint. Net photosynthetic rate is limited by stomata closure, which is controlled by turgor pressure of the leaf cells. The leaf turgor pressure can be highly affected by abscisic acid (ABA), which is responsible for the hydroactive stomatal closure under physiological drought. Water deficit enhances ABA generation in roots and xylem-mediated ABA transport to shoots. An increase in pH of xylem sap up-regulates the transport of ABA to the guard cells on leaf surfaces, where it regulates the stomatal closure through the involvement of  $\text{Ca}^{2+}$  (SHAHID et al.,



**Fig. 4:** Net CO<sub>2</sub> assimilation rate (Pn) and stomatal conductivity (Gs) in leaves of spinach plants exposed to high salinity, with or without triacontanol (TRIA) treatment. Ø stands for the control group. Vertical bars represent  $\pm$  standard error from means (n=5), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

2020). PANG et al. (2020) found that triacontanol inhibits ABA signal transduction with down-regulation of genes involved in the process, thus it compensates for stomatal closure induced by physiological drought caused by high salinity. By increasing the amount of the available carbon dioxide due to regulation of stomatal movements, TRIA can prevent excessive yield loss caused by high salinity in the rhizosphere. The maximal photochemical efficiency (also known as potential quantum yield) of photosystem II, indicated by the Fv/Fm chlorophyll fluorescence ratio, was not influenced by foliar spraying with 1  $\mu$ M triacontanol in spinach plants which were not exposed to salt stress (Fig. 5A). Fv/Fm of plants grown for twenty-one days in the presence of high salinity was markedly reduced. In plants exposed to 150 mM NaCl, treatment with 1  $\mu$ M TRIA totally counteracted the lowering effect of severe salt stress on this physiological marker of photosynthetic light use efficiency. Decrease of the potential quantum yield is an early indicator of disturbance of the light reactions of photosynthesis when environmental stressors impair energy conservation in plants, and TRIA significantly compensated for the inhibition effect of high salinity. BOROWSKI et al. (2000) observed a significant increase of the maximal quantum yield of PSII (Fv/Fm) and of the efficiency of excitation capture by open PSII reaction centers (Fv'/Fm'), as well as a reduction of non-photochemical quenching coefficient (qNP), when triacontanol was applied to tomato. Plants treated with triacontanol at the doses of 0.3 and 3.0  $\mu$ g had significantly higher yields of fruits than control. Improvement in

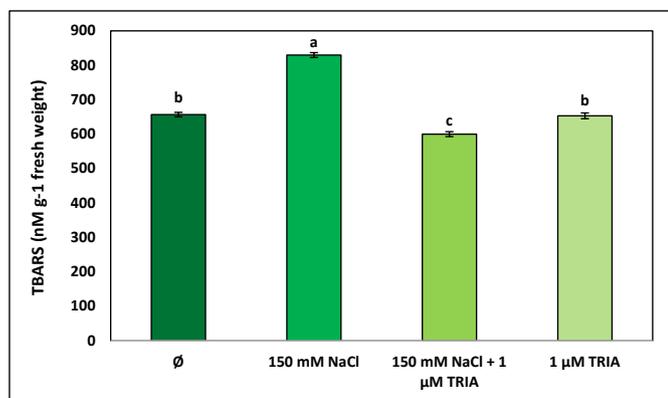
photosynthesis may be associated with increased chlorophyll content of spinach leaves. Plant pigments, particularly leaf chlorophyll contents have widely been appraised as indicators of photosynthesis and ultimately plant productivity. The fact that leaf spraying with TRIA causes a moderate, but statistically significant increase of the total chlorophylls in spinach leaves (Fig. 5B.) may be related to the fact that triacontanol stimulates the biosynthesis of chlorophyll pigments. CHEN et al. (2003) obtained similar results, in which case TRIA increased the contents of total chlorophylls by 25.1%, and Fv/Fm and effective quantum yield of photosystem II ( $\Phi$ PSII) were higher in the treated rice plants. PANG et al. (2020) demonstrated that exogenous application of TRIA stimulates expression of the genes encoding for protoporphyrinogen III oxidase (PPOX), chlorophyllide *a* oxygenase (CAO) and divinyl chlorophyllide *a* 8-vinyl-reductase (DVR), thus it plays a substantial role in chlorophyll biosynthesis. Another explanation for the better utilization of light energy in photochemical reactions under salt stress is due to the larger size and/or higher degree of organization of the light-harvesting antenna complexes (LHCII+LHCI), as well as a higher efficiency of excitation trapping at the reaction centers of PSII, caused by triacontanol.



**Fig. 5:** Potential quantum yield of photosystem II (Fv/Fm) and total chlorophyll content in leaves of spinach plants exposed to high salinity, with or without triacontanol (TRIA) treatment. Ø stands for the control group. Vertical bars represent  $\pm$  standard error from means (n=5), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

### Degree of oxidative membrane damage

The degree of oxidative membrane damage of salt-stressed spinach seedlings was shown by increased malondialdehyde (MDA) and related thiobarbituric acid-reactive substances (TBARS) content, these compounds being toxic products of fatty acid degradation. TBARS increased by 28% in plants exposed to 150 mM NaCl, compared to control seedlings. Simultaneous application of 1  $\mu$ M TRIA with salinity significantly reduced TBARS formation in spinach leaves (Fig. 6). Thylakoid membranes contain a high percentage of polyunsaturated fatty acids (mainly in monogalactosyl-diacylglycerol), thus very susceptible to lipid peroxidation. A slight perturbation in the composition of thylakoid membranes can lead to impaired photochemical reaction and energy coupling between reaction centers and antenna complexes. In the present study, it was found that exogenous application of 1  $\mu$ M TRIA mitigated the negative effects of salt stress on membrane lipid peroxidation and exhibited a protective capacity for biological membranes. Similar results were reported in case of salt-stressed coriander, when treatment with 10  $\mu$ M TRIA reduced H<sub>2</sub>O<sub>2</sub> and malondialdehyde formation by modulating the cellular redox status and the activity of antioxidant enzymes, such as superoxide dismutase and catalase (ASADI KARAM and KERAMAT, 2017). Inhibition of light-induced lipid peroxidation by TRIA was observed previously in isolated spinach chloroplasts (RAMANARAYAN et al., 2000). Under stressful conditions TRIA is able to increase the activities of antioxidant enzymes such as ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR),

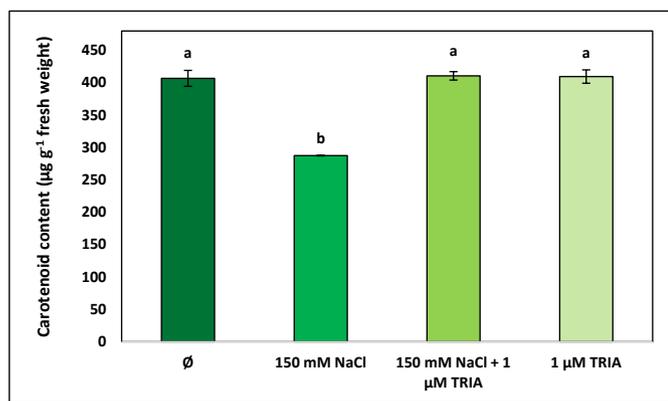


**Fig. 6:** Membrane lipid peroxidation assayed with the amount of thiobarbituric acid-reactive substances (TBARS) in roots of spinach plants exposed to salinity, with or without triacontanol (TRIA) treatment. Ø stands for the control group. Vertical bars represent  $\pm$  standard error from means ( $n=5$ ), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

so reducing the oxidative stress (ASADI KARAM et al., 2017a). In the human organism, malondialdehyde-modified low-density lipoprotein (MDA-LDL) acts as a marker of oxidative stress and is associated with atherosclerotic cardiovascular diseases, such as aortic stiffness (HOU et al., 2020). The current results support the idea that treatment with micromolar amounts of triacontanol as foliar spray may efficiently alleviate oxidative membrane damage induced by salt stress in spinach plants, thus reducing the malondialdehyde content in the human diet which contains this leafy vegetable.

### Carotenoid content of spinach leaves

Carotenoid content of spinach leaves is not influenced by the presence of triacontanol under stressless growth conditions. Salt stress generated by 150 mM sodium chloride decreased total carotenoid content by 29% (Fig. 7). Simultaneous application of 1 µM TRIA completely counteracted the deleterious effect of salinity on the carotenoid pigment concentration, thus restoring the health-promoting quality of these spinach leaves due to the antioxidative protective role of these carotenoids for every living organism. In the absence of salt stress, NAEEM et al. (2011) reported that exogenous application of TRIA significantly improved the carotenoids content of a mint species, exceeding the control by 26%. Carotenoids have two important

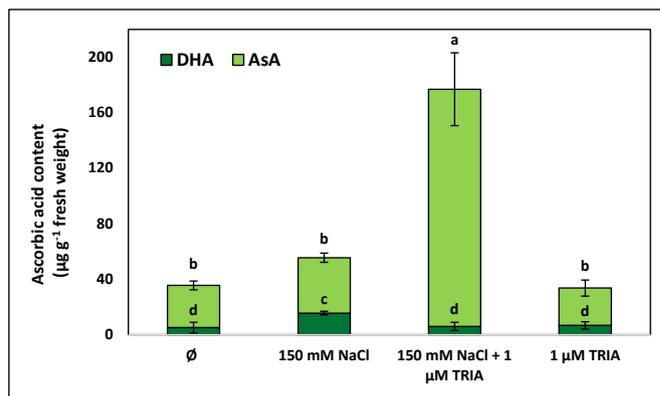


**Fig. 7:** Influence of triacontanol (TRIA) on carotenoid pigment content in leaves of spinach exposed to 150 mM NaCl. Ø stands for the control group. Vertical bars represent  $\pm$  standard error from means ( $n=5$ ), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

roles in photosynthetic organisms. Firstly, as light-harvesting pigments, they effectively extend the range of blue light absorbed by the photosynthetic apparatus, secondly, they play a crucial role in the dissipation of harmful excess excitation energy under stressful conditions which may impair the use of light energy in carbon assimilation. Based on their capacity to neutralize singlet oxygen, hydroxyl and organic peroxy radicals, carotenoids have a general protective role against oxidative damage. Humans cannot synthesize carotenoids and must ingest them in food or via supplementation. They primarily exert antioxidant effects, but carotenes also have a pro-vitamin A function, while lutein and zeaxanthin constitute macular pigments in the eye. The benefit of lutein in reducing progression of age-related macular eye disease and cataracts was proven experimentally. Plant-derived carotenoids also produce improvements in cognitive function and cardiovascular health, and may help to prevent some types of cancer (EGGERSDORFER and WYSS, 2018), therefore an increased carotenoid content of spinach plantlets upon human consumption has a demonstrated health-promoting role.

### Influence of foliar spraying with triacontanol on the vitamin C content

Ascorbic acid is one of the most abundant water-soluble antioxidants in plants. As plant-based foods constitute the principal source of vitamin C in the human diet, the possibility of increasing the ascorbate content of plants to improve their nutritional value has received considerable attention in recent years. This antioxidant molecule is considered as an essential component of cell signaling pathways triggering adaptive plant responses. Triacontanol by itself did not exert any significant influence on the vitamin C content of spinach leaves, when it was used in the concentration of 1 µM as foliar spray on the 'Popey' spinach cultivar. Salt stress exerted by 150 mM sodium chloride, with no TRIA treatment, slightly but significantly increased the ascorbic acid content of leaves, as a defense reaction to the oxidative stress induced by a prolonged exposure to high salinity. When treatment with 1 µM triacontanol was applied simultaneously with the high salinity, the vitamin C content was approximately four times as much as in control leaves (Fig. 8). Foliar application of TRIA increased not only the overall vitamin C content of spinach leaves, but it also led to an elevated AsA/DHA ratio in comparison to TRIA-untreated, NaCl-exposed plants. This shows an enhanced capacity to cope with oxidative stress, as the reduced ascorbate has the capacity to quench hydrogen peroxide, and its recovery from the oxidized forms is crucial for a sustained protective capacity. A similar role of



**Fig. 8:** Influence of TRIA on vitamin C content and on reduced ascorbic acid (ASC) to oxidized dehydroascorbate (DHA) ratio of spinach leaves exposed to salt stress. Ø stands for the control group. Vertical bars represent  $\pm$  standard error from means ( $n=5$ ), different letters above the columns indicate significant differences at  $P < 0.05$  (Tukey's HSD test)

S-methylmethionine (vitamin U) was demonstrated in lettuce seedlings exposed to salt stress induced by 150 mM NaCl (FODORPATAKI et al., 2016). Increment of the ratio between the reduced and the oxidized form of vitamin C is associated with a stronger antioxidative defense system and increased activity of monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) enzymes. MDHAR and DHAR activity increased by 39.11% and 273%, respectively, when canola seedlings were pretreated with 10  $\mu$ M TRIA before Cd-induced oxidative stress (ASADI KARAM et al., 2017b). These results clearly indicate the protective ability of TRIA to modulate the redox status of plant cells under unfavorable environmental conditions. However, the role of exogenously applied TRA in the ascorbic acid biosynthesis needs to be elucidated. Plants synthesize vitamin C in the highest amounts especially under environmental conditions which cause oxidative stress. Vitamin C cannot be produced by the human organism, so a systematic supply with this vitamin through alimentation is required for normal cell functions, for immune stimulation and synthesis of collagen. Severe deficiency leads to scurvy, whereas a limited vitamin C intake causes increased susceptibility to infections. Therefore, new strategies aimed to increase vitamin C content in plants would be of interest to improve human health.

### Conclusions

Treatments with very small concentrations of triacontanol is considered a profitable mean of augmenting quality and production of crop plants. In spinach, it improves vegetative growth and biomass production, and also stimulates seed germination. Under high salinity conditions, TRIA improves the nutritional value of spinach leaves, by inducing a higher carotenoid and vitamin C content, and by reducing the generation of toxic products of oxidative membrane damage (such as malondialdehyde). Adverse effects of high salinity of soil solution may be compensated for by very low amounts of TRIA pulverized on leaves. Improvement of photosynthetic functions was indicated by a more efficient net photosynthetic carbon dioxide assimilation as well as by a more efficient use more of the incident light energy when photosynthesis was impaired by salt stress. Development and production of crop plants may be improved by use of TRIA in an environmental-friendly approach. The present study can serve as a foundation for additional laboratory and field studies optimizing the application of triacontanol in crop production, as part of innovative cropping technologies. Our results may contribute to enhance yield and nutritional composition of crop plants cultivated in areas affected by increasing soil salinity, in relation with global climate change and with the need to ensure higher quantities and better qualities of horticultural products for a continuously increasing human population, in a hastily changing environment.

### Conflict of interest

No potential conflict of interest was reported by the authors.

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## ORCID:

Bernat Tompa  <https://orcid.org/0000-0002-3474-3203>Janos Balint  <https://orcid.org/0000-0002-0351-888X>Laszlo Fodorpataki  <https://orcid.org/0000-0003-2140-0137>

Address of the corresponding author:

Dr. Laszlo Fodorpataki, Department of Horticulture, Sapientia Hungarian University of Transylvania, RO-540485 Targu Mures, Romania

E-mail: [lfodorp@gmail.com](mailto:lfodorp@gmail.com), [fodorpataki.laszlo@ms.sapientia.ro](mailto:fodorpataki.laszlo@ms.sapientia.ro)

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