Chilling injury induction and water deficiency is accompanied by changes on the photosynthetic apparatus and antioxidant response in primary *Tagetes* leaves

A. GIANNAKOULA*[a], I. ILIAS[a] , A. PAPASTERGIOU[b], A. HATZIGAIDAS[b]
[a]Department of Crop Production, [b]Department of Electronics,
Technological Educational Institute of Thessaloniki,
PO Box 141, Sindos 541 00, GREECE

Abstract Plants are often subjected to the changes in temperature in their environment. Chilling stress is a complex phenomenon which reflects various changes in their function and structure. The destructive effect of chilling temperatures is visible not only in disturbances of physiological metabolic processes, but also in generating reactive oxygen species (H_2O_2, O_2^-, OH). These conditions of chilling stress (like other kind of stresses) results to an increase of active oxygen species (AOS) such as hydrogen peroxide. Water deficits is one of the common environmental factor limiting crop productivity. It brings about changes in membranes properties and produce O^*_2 and other reactive oxygen species. It is now becoming clear that oxidants are important in signal transduction processes. The high level of ROS in the cells of the plants is the reason of the occurrence of oxidative stress conditions there. The antioxidative system that includes low-molecular antioxidants (glutathione, ascorbate, cysteine) and antioxidative enzymes (superoxide dismutase-SOD, catalase-CAT, peroxidase-POX) is the mechanism protecting ROS. In the conducted research, we have demonstrate that H_2O_2 and peroxidase activity increased in leaves and especially when the plants were treated with low temperature and water deficit simultaneously. In this study also the changes in the levels of some stress markers (products of lipid peroxidation, of malondialdehyde equivalents, (MDA), hydrogen peroxide) and activities of stress defence enzymes (catalase, peroxidase, superoxide dismutase). The plants were grown under laboratory controlled conditions (growth chamber) as soil culture in plastic pots (30x30x10 cm). In the stress conditions the chlorophyll, protein contents significantly declined. Our results indicate specific responses of the antioxidant enzymes under stress conditions and a possible synergetic action of water stress and chilling causing intense changes in membrane properties, compared to samples exposed to either of the stresses individually. We concluded that it is the concerned action of antioxidants, and not an antioxidant alone, which confers protection from oxidative stress in chloroplasts of chilling stressed plants.

Keywords: chilling, environment, soil content, water stress, antioxidant enzymes, malondialdehyde,

1. Introduction

Plants adapted to habitats characterized by wide ranges of spatial and/or temporal variation tend to exhibit a predisposition for photosynthetic acclimation (i.e., phenotypic responses to environmental change), enabling them to physiologically compensate for large shifts in abiotic parameters (e.g., irradiance and temperature; reviewed by Berry and Bjorkman,1980). Low temperature reducesthe capacity of photosynthetic system to utilise incident radiation, leading to a photoinhibition process and characterized by over- excitation of thylakoid membranes and subsequent impairement of photosynthetic function (Jung and Steffen,1997). A plant biological response to temperature stress is depending on a number of factors, including species, crop development stage, and environmental conditions (Heck and Miller, 1994). The biological response is culmination of a series of physical,

biochemical and physiological events, beginning with changes in the rate of photosynthesis in stomatal conductance and tranpiration rate. The decreased efficiency of PSII photochemistry under stress conditions may reflect not only the inhibition of PSII function (can be measured as biological responses) but also modification of level of the antioxidants metabolites or alteration in the activity of antioxidants enzymes.

The physiological responses of plants to water availability have long been of interest, mainly because of the need to understand better the effects of economically important plants when water is a limiting factor. A reduction in the efficiency of photosynthesis is among the biochemical manifestation of water stress (Dubey, 1999). A direct effect of low water availability in higher plants is the stomatal closure (Alexander et al., 1995). It brings about changes in membrane properties, marked by decrease in the level of either phospholipids and galactolipids or total fatty acids (Linjenberg, 1992). However, photosynthetic electron transport is maintained at a relatively faster rate in the stressed leaves as compared to the higher decrease in the rate of CO_2 fixation (Baisak et al., 1994). Also because of stomatal closure, CO_2 fixation is limited and there is a decreased availability of oxidized $NADP^+$ as an electron acceptor for photosystem I. This imbalance between electron transport and CO_2 fixation rates may result in the over-reduction of the electron transport chain components and facilitate the transfer of electron to O_2 to produce $^1O_2^*$ H_2O_2, highly toxic hydroxyl radicals ($^*OH$) (Larson, 1988) and other reactive oxygen species (Minkov et al., 1999). The reactive oxygen species formed, also cause membrane distortion, leakage of electrolytes via lipid peroxidation (Egert and Tevini, 2002), overall inhibition of protein synthesis resulting in DNA damage, inactivation of chloroplastic enzymes, impairment of electron transport, increased membrane permeability etc. (Aziz and Larher, 1998). This indicates that the effect of water stress on plants is extensive.

On the other hand, plants do not appear equally sensitivity chilling exposure at all stage in their life cycle. The objective of this study was to determine the physiological and biochemical effects of low temperature and water deficiency for a period of 12 days, on CO_2 assimilation, transpiration rate, stomatal conductance and chlorophyll fluorescence in relation to peroxidase activities and MDA content in *Tagetes* plants and to define early warning processes prior to the detection of yield effects. *Tagetes* is a widely grown in the during the winter growing season and its production has great economic and commercial values. The results presented demonstrate that simultaneously induced chilling and drough stress: a) resulting in inhibition of photosynthesis and increase in the activity of antioxidant enzymes; b) caused less inhibition of photosynthesis compared to seperately induced drough stress.

2. Material and Methods

2.1 Growth conditions

Seeds of *Tagetes* sp. were sown in plastic pots (30x30cm) containing a mixture of commercial potting soil and vermiculite (2:1). Seed germination and plant growth were conducted in a controlled growth chamber under a 16h/8h and 22^{o}C /20^{o}C dark/light cycle with an irradiance of 295 μmol m^{-2} s^{-1}, illumination was provided by a combination of fluorescent (Osram, Fluora-Plant Lighting, L58W/77) and incandescent (General Electric, Neodymium R80, 100W) lamps. Relative humidity varied between 79.4±6.2 and 65±3.2%. Seven-day-old seedlings were tranplanted in larger plastic pots (10 dm^3 capacity). Seedlings were supplied every second day with modified Hoarland nutrient solution (pH 5.8) on all remaining days until the onset of the chilling and drought stress treatments.

2.2 Stress treatments

Experimentation began when the plants were about 10-d old plants and had four leaves. In the beginning of each experiment leaf 3, counted from the bottom, was tagged for use in physiological and biochemical analysis.

These leaves had reached full expansion. Four irrigation treatments were applied in the same way. Two- group of plants were continuously received: nutrient solution irrigation treatment and non-irrigation treatment at 21^0C. The other two-group of plants were also received: nutrient solution irrigation treatment and non-irrigation treatment at 5^0C.

2.3 Gas-Exchange Measurements

Gas-exchange measurements were made from the same leaf with a portable photosynthetic apparatus Li-6200 (Licor, Inc., Lincoln, USA) with IRGA. The carbon dioxide (CO_2) analyzer was calibrated with two standard CO_2/air mixtures. The lamina of the leaf was enclosed within a fun stirred ¼ L cuvette. The mean CO_2 concentration and leaf to air vapour pressure deficit for all measurements were 350 μmol mol^{-1} and 20 mbar respectively. Measurements were made at 25°C under a photon flux density of about 600 μmol m^{-2} s^{-1}. Calculations of A (CO_2 assimilation rate), E (transpiration rate), Ci (CO_2 intercellular concentration) and g_s (stomatal conductance) from gas-exchange measurements were according to Von Caemmerer and Farquhar (1981).

2.4 In Vivo Chlorophyll Fluorescence Measurements

In Vivo Chlorophyll fluorescence was measured on the upper surface of the third fully expanded leaf, after being left for 30 min to dark adaptation, at room temperature. The chlorophyll fluorescence induction curve monitored by a Plant Analyser (PEA, Hansatech Ltd King's Lynn, Norfolk, England) with 600Wm^{-2} of red (630nm) light intensity (excitation intensity) (Pereira et al., 2000). The initial fluorescence intensity (Fo) when all reactions centres (RCs) are open, Fm maximal fluorescence intensity when all reactions centres (RCs) are close, F_v variable fluorescence, t_{max} time to reach the maximal fluorescence intensity were calculated. The indicators were measured at room temperature on intact leaves of four replicate plants from the four treatments.

2.5 Determination of lipid peroxidation

The level of lipid peroxidation in shoots/leaves after five days of chilling stress and 3 days of recovery treatment was measured as malondialdehyde (MDA) content determined by reaction with 2-thiobarbituric acid (TBA) reactive substances according to Heath and Packer (1968). The tissue was homogenized in 0.3% TBA in 10% trichloracetic acid (TCA) at 4 °C. The concentration of MDA was calculated from the difference of the absorbance at 532 nm and 600 nm using the extinction coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as nmol (MDA) g^{-1} of fresh weight.

2.6 Extraction of enzymes

Soluble peroxidase activity (PA) were performed in 3-week old leaves. Samples were taken at day 0, 3, 6, 9, of each irrigation treatment, lyophilized and homogenized (extraction ratio 1:10) for two min in a mortar with two ml Tris-HCl buffer (0.1M, pH 7.5) containing 1mM Phenyl methyl sulfonic fluoride(PMSF) and 5% (w/v) Polyvinyl-polypyrolidone (PVPP) in the presence of liquid nitrogen. The homogenate was centrifuged at 10.000xg for 15 min at 4 °C. The supernatant was dialyzed against extraction buffer (except PVPP) and was used as a soluble peroxidase source.

2.7 Peroxidase assays

Total peroxidase activity was determined spectrophotometrically by monitoring the formation of an indamine dye from 3-dimethylamino benzoic acid (DMAB) AND 3-methyl-2-benzothiazolinone hydrozone hydrochloride monohydrate (MBTH) at 590 nm in the presence of H_2O_2 according to the method of Ngo and Lenhoff, (1980).

2.8 Protein estimation

The sodium chloride soluble proteins were estimated by Coomassie brilliant blue G-250 method as described by Bradford (1976). Bovine serum albumin was used as standard.

3. Results and Discussion

The effect of the different stress treatments on PA (peroxidase activity, ΔAbs min^{-1} mg^{-1} protein) is shown in the Table 1. The extractable specific activity of PA from untreated leaves was almost unchanged from day 0 to day 12. No increase in activity was observed in chilling stress after 3 or 6 days, while ther was observed an increase (68%, $p \leq 0.05$) in activity afer 9 days of stress treatment .No increase in activity was also observed the first 3 days of any of the stress treatments. With regard to chilling + drough stress treatment, a 80% increase was noticed on the ninth day.

Untreated	0 day	3rd day	6th day	9th day
	0.25± 0.01	0.25± 0.05	0.25± 0.08	0.27± 0.01
Chilling	ns	ns	ns	0.40± 0.04*
Chilling +Drought	ns	ns	0.50± 0.08*	0.89±0.08*
Drought	ns	ns	0.48± 0.04*	0.77± 0.05*

Table 1. Increase in the specific activity of peroxidase extracted from leaves (n=3) harvested at the end of every day of exposure in plants subjected to chilling and drought stress. (ns= no significant increase and *=$p \leq 0.05$).

CO_2 assimilation rate (A) concerning all the stress treatments was reduced about 20% for the chilling stress treatment and about 35% for the drought stress treatment, while in both stress treatments the assimilation rate was reduced sharply (80% reduction) (Table 2). Table 2 also shows the values of transpiration rates (E), stomatal conductance (g_s) and CO_2 intercellular concentration (Ci). The highest transpiration rates values were observed in the nutrient irrigated under no chilling treatment (control). Transpiration rates in the chilling + drought stress treatments were 20% lower in comparison to respective control (Table 2).

The highest stomatal conductance (g_s) and CO_2 intercellular concentration (Ci) values were observed in chilling + drought stress treatments (60%) compared to control. Chilling stress and drought stress negatively affected CO_2 assimilation rate (A) but the lower value was recorded in the presence of both stresses. Reduction of CO_2 assimilation rate can be partly related to stomatal closure perfomance of which is determined by the conditions at or in the root system (Pankovic et al., 1999). Ci measurements during chilling + drought stress treatments significantly increased. This is apparently the effect of parallel changes in stomatal condyctance and photosynthetic activity. This means that in Tagetes leaves CO_2 assimilation is limited both by stomatal closure and mesophyllic CO_2 fixation ability.

Furthermore the observation that water deficiency in the root area enhases the stomatal closure is well established (Shone and Flood, 1980). The lowest transpiration rate (E) values were observed in the chilling +drought stress treatments possible related to a low vapor pressure deficit, since transpiration rate is primarily a function of vapor pressure gradient and leaf stomatal conductance (Alexander et al., 1995). The same low valuesin stomatatal conductance also presentented

	Amax	E	g_s	Ci
Untreated	15.4a	3.3a	0.42a	273.4a
Chilling	12.7a	2.87a	0.27b	268.4a
Chilling +Drought	3.5b	2.1b	0.16c	339.6b
Drought	9.2a	2.4a	0.22b	245.1a

the chilling +drought stress treatments in comparison with the control. This decline must have been resulted from the direct effect of water deficiency on the stomatal guard cells. Lower stomatal conductance values also reported in other plants in extreme environments (Gimenez et al., 1992).

Table 2. Assimilation rate (A, μmol CO_2 m^2s^1), Transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$), stomatal conductance (g_s, mol H_2O $m^{-2}s^{-1}$) and CO intercellular concentration(Ci,μmol CO_2 $m^{-2}s^{-1}$) on 12th day of stress treatments. Values are means of five samples. Values followed by the same letter indicate no significant differences.

The stomatal closure and the reduction of CO_2 assimilation rate, observed during drought stress, are often followed by an increase of peroxidase activity (Shone et al., 2001; Vinit-Dunand et al., 2002). Under water stress conditions the concentration of O^-_2 and H_2O_2 increased since the stomata get upset and close

(Larson, 1988). As a result, a significant increase in the content of antioxidant enzymes, such as peroxidases was noticed (Larson, 1988; Egert and Tevini, 2002;Yordanov et al., 2000). These enzymes scavenge part of ROS and protect the photosynthetic machinery (Egert and Tevini, 2002; Rios-Gonzalez et al., 2002; Candan and Tarhan, 2003). Under similar stress conditions, such as water stress and chilling stress conditions, the peroxidase activity (PA) increased significantly. The above increase of PA could be due to the response to synergetic action of water and chilling stress.

In this study chilling +drought stress induced a decrease in A and g_s of the *Tagetes* plants and alteration in Ci. A possible explanation can be put for these data: stomatal closure Similar conclusions have been reached by other authors in other plants (Vu and Yelenosky, 1991). Stomatal closure may involve disturbances in electron transport as indicated by the study of leaf fluorescence. Measurements of chlorophyll fluorescence showed that reductions in A due to chilling +drought stresses were associated with a decrease in Fv/Fm ratio. The Fv/Fm ratio, which indicates the photochemical efficiency of PSII, change in the leaves of both simultaneously stressses as well as Fv/Fo ratio.These reductions demonstrated that photochemistry of PSII, light-driven electron transport and enzymatic reactions requiring ATP and NADPH from chloroplasts were significantly affected by both stresses. Since PSII efficiency appeared to be affected , CO_2 availability at the carboxylation sites may not be the only determinant for reduced CO_2 fixation. The CO_2 transfer resistance from intercellular spaces to the chloroplastic stroma is not clearly understood. Stuhlfauth et al., (1990) highlighted a decline of chloroplastic CO_2 concentration, coupled with a maintenance of a constant calculated Ci in some species. Cornic, (1994) reported that in leaves stressed by drought, the resistance to diffusion of CO_2 from the cell wall to the chloroplast may be the major limiting factor.

Lipid peroxidation was altered significantly The above increase of MDA could be due to the response to synergetic action of water and chilling stress.

	Fv/Fo	Fv/Fm	MDA
Untreated	3.623a	0.782a	1.09± 0.25
Chilling	3.170a	0.754a	1.55± 0.07
Chilling +Drought	2.654b	0.718b	1.99±0.15**
Drought	2.586b	0.734b	1.75± 0.11*

Table 3. Chl fluorescence parameters and MDA(μmol g^{-1} f.w.) content on 12^{th} day exposed to single or simultaneously stresses. Each value is the mean of five replicates. For each column, means flanked by the same letter are not significantly different. Values expressed as means± SE (n=5). Significantly different from controls at $p \leq 0.01$ (**) and $p \leq 0.05$ (*)

4. Conclusions

Tagetes cultivars are sensitive to water stress and chilling stress simultaneously. As a result reduction of CO_2 assimilation rate (A), transpiration rate (E) and stomatal conductance (g_s) values were observed. The changes of the photosynthetic parameters, seems to be related with an increase of peroxidase activity and lipid peroxidation. Furhermore, there was observed a reduction of plant growth and flower production and deterioration of flower quality.

Aknowledgements

The project is co-funded by the European Social Fund & National Resources-EPEAK II- ARCHIMIDIS

References

1) **Alexander J.D., Donnelly J.R., Shane J.B., 1995.** Photosynthetic and transpirational responses of red spruce understory trees to light and temperature. Tree Physiol. 15: 393-398.
2) **Alscher J.D., Donahue J.L., Cramer C.L., 1997.** Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant 100: 224-233.
3) **Aziz and Larhen, 1998.** Osmotic stress induced changes in lipid composition and peroxidation in leaf discs of Brassica napus L..J Plant Physiol 153:754-762.
4) **Baisak R., Rana D., Acharya P.B., Kar M., 1994.** Alterations in

the activities of active oxygen scavenging enzymes of wheat leaves subjected to water stress. Plant Cell Physiol 35: 489-495.

5) **Berry J. and Björkman O., 1980.** Photosynthetic response and adaptation to temperature in higher plants. *Annu. Rev. Plant Physiol.* **31** (1980), pp. 491–543.
6) **Bradford M.M., 1976.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Anal. Biochem. 72: 248-254.
7) **Cornic G., 1994.** Drought stress and high light effects on leaf photosynthesis. In Photoinhibition of photosynthesis, 17, Baker N.R. and Bowyer J.R., ed., Bios Scientific Publisher, 297-313.
8) **Dubey R.S., 1999.** Protein synthesis by plants under stressful conditions. In Pessarakli M(ed) Handbook of Plant and Crop Stress, 2nd edition. Marcl Dekker, New York, pp 365-397.
9) **Egert M. and Tevini M., 2002.** Influence on some physiological parameters symptomatic for oxidative stress in leaves of chives (Allium schoenoprasum). Env. Exp. Bot. 48: 43-49.
10) **Gimenez C., Mitchell V.J., Lawlor D.W., 1992.** Regulation of photosynthetic rate of tho sunflower hybrids under water stress. Plant Physiol. 98: 516-524.
11) **Heck W.W. and Miller J.E., 1994.** Airpollution: plant growth and productivity. In: Arntyen CJ (ed) Encyclopedia ofAgricultural Science, Vol. 1. Academic Press, San Diego, CA, pp 27-39 ISBN 0-122-26670-6.
12) **Larson R.A., 1988.** The antioxidants of higher plants. Phytochemistry 27: 969-978.
13) **Liljenberg C.S., 1992.** The effects of water deficit stress on plant membrane lopids. Prog Lipid Res 31: 335-343.
14) **Minkov I.N., Jahoubjan G.T., Denev I.D., Toneva V.T., 1999.** Photooxidative stress in higher plants. . In Pessarakli M(ed) Handbook of Plant and Crop Stress, 2nd edition. Marcl Dekker, New York, pp 499-525.
15) **Ngo T. and Lenhoff M., 1980.** A sensitive and versatile chromogenic assay for peroxidase and peroxidase coupled reactions. Anal. Biochem. 105: 389-397.
16) **Jung S. and Steffen K.L.,1997.** Influence of photosynthetic photon flux densities before and during long-term chilling on xanthophyll cycle and chlorophyll fluorescence quenching in leaves of tomato (*Lycopersicon hirsutum*). Physiologia Plantarum 100: 958-966.
17) **Pankovic D., Sakac Z., Kevresan s., Plesnicar M., 1999.** Acclimation to long term water deficit in the leaves of two sun flower hybrids: photosynthesis, electron transport and carbon metabolism. J. Exp. Bot. 50: 127-138.
18) **Smirnoff N., 1993.** The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125: 27-58.
19) **Shone M.G.T., Flood A.V., 1980.** Studies on uptake and loss of water by barley roots in relation to changes in root resistance. J. Exp. Bot. 31: 1147-1151.
20) **Stuhlfauth T., Scheuermann R. and Fock H.P.,1990.** Light energy dissipation under water stress condition. Contribution of reassimilation and evidence for additional processes. Plant Physiol. 92: 1053-1061.
21) **Vinit-Dunand F., Epron D., Alaoui Sosse B., Babot P-M., 2002.** Effects of copper on growth and on photosynthesis of mature and expanded leaves in cucumber plants. Plant Sci. 163: 53-58.
22) **Vu J.C., Yelenosky G., 1991.** Photosynthetic responses of citrus leaves to soil flooding. Physiol. Plant. 81: 7-14.