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STSM Applicant: Dr Damianos Neocleous, Agricultural Research Institute, Nicosia (CY),

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STSM topic: “Antioxidant response of strawberry plants under stress conditions”

Date of STSM: 26/01/2009-27/02/2009. The applicant was engaged in a training research program from the 29th December 2009 to 27th February 2009.

Objective: The aim of this STSM was to make measurements using methods not available in my home institution and learn the laboratory skill involved in the quantification of enzymatic and nonenzymatic antioxidant-molecules. Improving my knowledge and understanding the physiological response of strawberry plants under stress conditions will benefit my present research and the work involved in our present Cost-Action helping me studying in a holistic approach the feasibility of an altered stress response of strawberry plants. This may facilitate as a tool to define agronomic practices to enhance yield under inverse environmental conditions and to obtain berry crops with high nutritional value, without heavily affecting fitness.

One of the biochemical changes occurring when plants are subjected to biotic or abiotic stresses is the production of reactive oxygen species (ROS) highly reactive and able to undertake catalytic functions in the absence of an energetic antioxidant mechanism (Foyer and Noctor, 2005). This ability means that stress-tolerant plants should have a better antioxidant system and induce changes in their secondary metabolites synthesis which are evolved by plants as a natural defence response under biotic and abiotic stress conditions (Kataoka, Beppu, Sugiyama, & Taira, 1996).

Finally, the experience and learning opportunities from the current STSM will help me to develop skills and expand my knowledge in developing, designing, conducting, analyzing, and reporting scientific research. In addition to achieving the goals of my mission, the visit certainly strengthened existing network.

Materials and Methods

Experiment 1 {Antioxidant response of strawberry plants under NaCl stress}

Treatments, plant material and growth conditions

Experiment was carried out at the Aristotle University of Thessaloniki experimental farm, Greece, during the period March-June 2008. Commercial frigo-strawberry plants (*Fragaria x ananassa* ‘Camarosa’), were cultivated in a non-heated greenhouse under soilless cultivation. The plants were planted in 3 L plastic pots filled with sterilised peat and perlite (1:1 v:v) as the planting medium. The plants were selected in order to facilitate a uniform plant material and irrigated interchangeably with 1/3 Hoagland nutrient solution (Hoagland and Arnon 1950) and water keeping the amount of drainage water at 20%. To improve vegetative growth all the runners were removed. The recorded average temperature and relative humidity inside the greenhouse was 20°C and 55% respectively. In late May the plants placed outside the greenhouse and supplied for 30 days with 1/3 Hoagland nutrient solution (pH~6.0) containing 0, 10, 20, and 40

mM NaCl equivalent to EC 1.2, 2.6, 3.9 and 6.6 mS cm⁻¹ respectively (NaCl stress). The solution, including the added NaCl was applied to the plants at sufficient rates until drainage to ensure salt stress in the root medium and replenishment of the various elements. The recorded mean temperature and relative humidity outside the greenhouse was 22°C and 58% respectively. The youngest fully expanded leaves were harvested, immediately frozen in liquid nitrogen and placed in polyethylene bags and stored. Results are the mean value of three consecutive harvests at 10th, 20th, and 30th day. At the end of the salt treatment period (30th day), roots samples from each plant were removed carefully washed with rainwater to remove any surface particles, frozen and stored. All samples were stored at -30°C till analysis (current STSM).

Experiment 2 {Antioxidant response of strawberry plants under drought induced by polyethylene glycol}

Treatments, plant material and growth conditions

The effects of drought stress induced by polyethylene glycol, PEG (molecular mass 4000) on some biochemical characteristics of strawberry were studied. Experiment was carried out at the Aristotle University of Thessaloniki, School of Agriculture, Greece, during the period January-February 2009. Freshly dug strawberry plants (*Fragaria × ananassa* Duch., cv. 'Camarosa') with one well-developed crown 8–10 mm in diameter were selected for hydroponic culture in a culture room at 22±2°C temperature with 16 h photoperiod under a light density of 150 μmole m⁻² s⁻¹. Firstly, plants were cultivated in aerated distilled water and then in aerated Hoagland's nutrient solution for 15 d. After this period, the media either remained unsupplemented (control plants) or were supplemented with 150 and 300 g/L PEG 4000. Young plants (with only two young leaves) were harvested after 24h of PEG treatment and leaves and roots were carefully washed with distilled water, frozen and stored at -30°C. Plants were damaged at the high PEG concentration and samples were not taken from this treatment.

Analyses performed during the STSM period: 26/1/2009 to 27/2/2009

Ascorbic acid (vitamin C)

Frozen samples were grounded and homogenized for 1 min. 5 g of the homogenized sample was added to 50 ml extracting solution (oxalic acid 1%) and homogenized for 1 min and filtered through Watman (No.1) filter paper. If decolorizing was necessary, 500 mg PVPP (polyvinylpolypyrrolidone) and some drops of H₂SO₄ (25%) were added in 10 ml of the filtrate. After allowing the PVPP to be deposited, the ascorbic acid content was measured with Reflequant system in combination with a standard solution into the supernatant. (Merck, Darmstadt, Germany).

Total phenolics and antioxidant capacity

Extraction

Samples (1 g) were extracted with 20 ml of a mixture containing acetone, water and acetic acid (70:29.5:0.5). The mixture after shaking vigorously was stored at 4°C for 24h, then filtered and the volume was brought up to 25 ml in a volumetric flask (Kähkönen et al., 2001; Asami et al., 2003).

Total phenolics

The content of total phenolics was measured according to Scalbert et al. (1989) with slight modifications. Briefly, 0.50 ml of diluted extract was mixed with 2.5 ml of 1:10 diluted Folin-Ciocalteu's phenol reagent followed by addition of 2 ml sodium carbonate (Na₂CO₃, 75 g L⁻¹). After shaking vigorously, the mixture was incubated at 50°C for 5 min, cooled and the absorbance measured at 760 nm. Gallic acid (G.A) was used as standard and the results expressed as mg g⁻¹ of G.A. in FW. Triplicate determinations were made in each sample.

Antioxidant capacity

The antioxidant capacity was measured using FRAP assay (Benzie and Strain 1996, 1999). Briefly FRAP reagent was freshly prepared to contain 0.3 M of sodium acetate at pH 3.6, 10 mM

2,4,6-tripyridyltriazine (TPTZ) in 40 mM HCl and 20 mM ferric chloride corresponding to 10:1:1. A 100 μ l of diluted extract was added to 3 ml of the FRAP reagent and mixed. After incubation for 4 min at 37°C, absorbance at 593 nm was measured against a water FRAP⁻¹ blank. Ascorbic acid (A.A) was used as standard and the results were expressed as μ mol g⁻¹ in FW. Triplicate determinations were made on each replicate value.

Total carotenoids

Root samples (1 g) were extracted with 20 ml of a mixture containing hexane/acetone/ethanol (2:1:1 v:v:v), and the total carotenoids concentrations in hexane layer were determined spectrophotometrically. Absorbance at 450 nm was measured in the supernatant after storing for 24 hours at 4°C in darkness according to the method described by Kuti (2004) with minor modifications, using the molar extinction coefficient of β carotene $E^{1\%} = 2592$ (Rodriguez-Amaya, 1999).

PAL activity

For determination of PAL activity, about 1 g of roots was homogenized in 10 ml, 100 mM borate buffer (pH 8.8) containing 2 mM EDTA, 5 mM 2-mercaptoethanol and 10 g L⁻¹ of PVPP. The homogenate was stirred for 1h at 4°C and then was centrifuged at 12 000 g for 20 min at 4°C. The supernatant was immediately assayed for PAL activity by the method reported by Zucker (1965). The reaction mixture (6 ml), containing 0.03 M borate buffer (pH 8.8), 0.01 M L-phenylalanine and 2 ml of enzymatic extract, was incubated at 40°C for 1 h (Jouili and Ferjani, 2003) and the reaction was terminated with 4N HCl. PAL activity was expressed as change in OD_{290 nm} h⁻¹ (g FW)⁻¹. One Unit of activity is defined as an increase in absorbance at 290 nm of 0.01.

Extraction and assay of peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD)

For POD and CAT assays, enzymes were extracted from 300 mg of tissue by grinding them in a cold mortar and pestle with the 1 ml 50mM K-phosphate buffer at pH 6.5 containing 1 mM EDTA, 2.0% (w/v) PVPP, 1 M NaCl, 0.05% Triton X-100 and 1 mM PMSF. For SOD extraction a mixture of 50 mM K-phosphate buffer (pH 7.6) containing 0.1 mM EDTA, 2% (w/v) PVPP, 0.05% Triton X-100 and 1 mM PMSF. The homogenate was centrifuged (15,000 \times 20 min at 4 °C) and the supernatant was used the antioxidant enzymes assays.

Peroxidase activity (soluble and ionically bound to cell wall) was determined according to Ngo and Lenhoff (1980). Adequate amounts of enzyme extract (100 μ l) were applied in a total reaction mixture 3 ml containing K-phosphate buffer 0.1 M (pH 6.5), 0.2 mM 3-methyl-2-benzothiazolinonhydrazon hydrochloride hydrate (MBTH), 10 mM 3-dimethylamino benzoic acid (DMAB) and of 0.3 mM H₂O₂. Absorbance changes were recorded at 590 nm with a *Shimadzu UV-1601* spectrophotometer (*Shimadzu*, Kyoto, Japan) at room temperature (25 \pm 2 °C). One unit of peroxidase activity (U) was defined as the increase of one unit of absorbance per minute, under the assay conditions and the enzymatic activity was expressed per fresh weight (U/min/g FW).

CAT activity (U) was determined by the decomposition of H₂O₂ at pH 7.0 and 25 °C according to Cakmak et al. (1993). The assay mixture contained 50 mM K-phosphate buffer (pH 7.0), 100 μ l of enzyme extract and 10 mM H₂O₂. The total reaction volume was 1 ml. The rate of change in absorbance (OD) at 240 nm was measured by spectrophotometer (*Shimadzu UV-1601*) and the levels of enzyme activity were expressed per minute per g fresh weight. (U/min/g FW)

SOD activity was assayed photochemically based on the photoreduction of nitro blue tetrazolium (NBT) by light in the presence of riboflavine and methionine (Beauchamp and Fridovich, 1971). NBT is reduced to blue diformazan, which has a strong absorbance at 560 nm under aerobic conditions SOD inhibits the formation of blue diformazan. The reaction mixture 3 ml contained 50 mM K-phosphate buffer (pH 7.8), 6.6 mM EDTA, dissolved in 0.3 mM KCN, 3 mM methionine, 0.05 mM NBT and 100 μ l of the enzyme extract. The reaction was initiated by adding 0.002 mM riboflavine and illuminating the mixture was with fluorescent lamp (15 W for 5 minutes). The the absorbance was determined at 560 nm compared to the enzyme free control.

One unit of SOD was defined as the amount of enzyme that yielded a 50% inhibition of the reduction of NBT. Results expressed per g FW.

Hydrogen Peroxide

Hydrogen peroxide was determined using the DMAB-MBTH system according to Ngo and Lenhoff (1980). For the extraction of hydrogen peroxide 500 mg of tissue were homogenized, by grinding them in a cold mortar and pestle, with the 1.5 ml HClO₄ (5%) and incubated for 5 minutes at 0°C. The homogenate was centrifuged (15,000 × 15 min at 4 °C) and the supernatant was used. HClO₄ was neutralized with HEPES/KOH 1M pH 7 and K₂CO₃ 1.7 M. 450 µL of the extract were neutralized with 150 µL HEPES/KOH and required amounts of K₂CO₃ up to pH 5.6. The mixture was centrifuged (15,000 × 15 min at 4 °C) and the supernatant was used. For the determination of hydrogen peroxide the total reaction volume was 3 ml. The assay mixture contained 0.2 mM MBTH, 10 mM DMAB, 0.1 M phosphate buffer (pH 6.5), 100 µL sample extract. The reaction was initiated by adding 10 µL POD (0.01 U) and was allowed to proceed at 25°C. The change in absorbance at 590 nm was continuously recorded and related with a standard curve of hydrogen peroxide to estimate the concentration per g fresh weight.

Proline

Proline was measured as described by Bates et al. (1973). 500 mg of fresh plant material was homogenized in 10 ml of 3% sulphosalicylic acid and the residue was removed by centrifugation. 1.5 ml of the extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) for 1 h at 100 °C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene. The chromophore-containing toluene was warmed to room temperature and its optical density was measured at 520 nm. The amount of proline was determined from a standard curve and results expressed per g fresh wt.

Protein content was estimated by the method of Bradford (1976), using BSA as a standard.

Results and Discussion

Experiment 1

In our experiment, the increase of antioxidant capacity in leaves imposed by salinity was presumably the result of raised total phenolics levels (Table 1). In roots, in addition to phenolics, ascorbic acid contributes to the antioxidant activity significantly (Table 2). Salt stress also activated PAL (Table 2), which is involved in the key steps of secondary metabolism. However, peroxidase activity did not show any significant differences compared to the control (Fig. 1). Alteration of antioxidants in leaves and roots, suggest that it is possible that in different strawberry tissues different mechanisms are involved in the protection against oxidative stress. Moreover, nonenzymatic antioxidative system is still intact and functioning enabling plants to respond to stress conditions. Compared with roots, leaves were found to have higher values of antioxidant molecules suggesting a higher antioxidant activity. In summary, our results suggest that the production of antioxidant substances may be stimulated by a moderate salt stress and this is of practical relevance for strawberry cultivation in hydroponics systems where the EC value could be elevated or in soils with slight salinization and in areas where the quality of water is not so good. Nevertheless, more extensive measurements are needed to assess long-term potential hazards associated with irrigation using saline water.

Table 1. The effect of salinity (NaCl concentration) on ascorbic acid (vitamin C), total phenolics and antioxidant capacity (FRAP values) of strawberry leaves.

Treatment (mM NaCl)	Ascorbic acid (mg (g FW) ⁻¹)	Total phenolics (mg GA (g FW) ⁻¹)	FRAP values (μmol (g FW) ⁻¹)
Salinity			
0	1,42 a	32,4 a	337 c
10	1,45 a	38,9 b	400 b
20	1,51 a	42,1 b	424 b
40	1,54 a	44,2 b	440 b

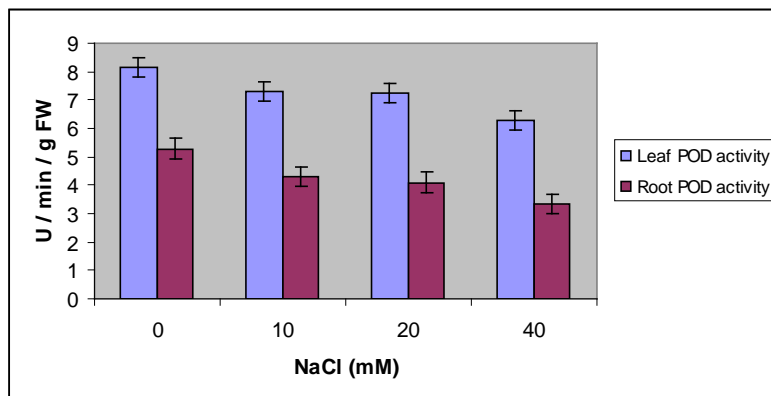
Means within a column for each NaCl level followed by different letters are significantly different according to Duncan's multiple range test at P<0.05 level.

Table 2. The effect of salinity (NaCl concentration) on carotenoids, ascorbic acid (vitamin C), total phenolics, antioxidant capacity (FRAP values) and PAL activity of strawberry roots

Treatment (mM NaCl)	Total Carotenoids (μg (g FW) ⁻¹)	Ascorbic acid (mg (g FW) ⁻¹)	Total phenolics (mg GA (g FW) ⁻¹)	FRAP values (μmol (g FW) ⁻¹)	PAL activity U (g FW) ⁻¹
Salinity					
0	20,6 a	0,34 b	7,54 c	51,6 b	3,59 b
10	17,2 b	0,39 a	9,43 ab	66,5 a	4,08 b
20	12,4 c	0,38 a	9,20ab	63,2 a	8,12 a
40	13,7 c	0,40 a	9,80 a	66,5 a	7,92 a

Means within a column for each NaCl level followed by different letters are significantly different according to Duncan's multiple range test at P<0.05 level.

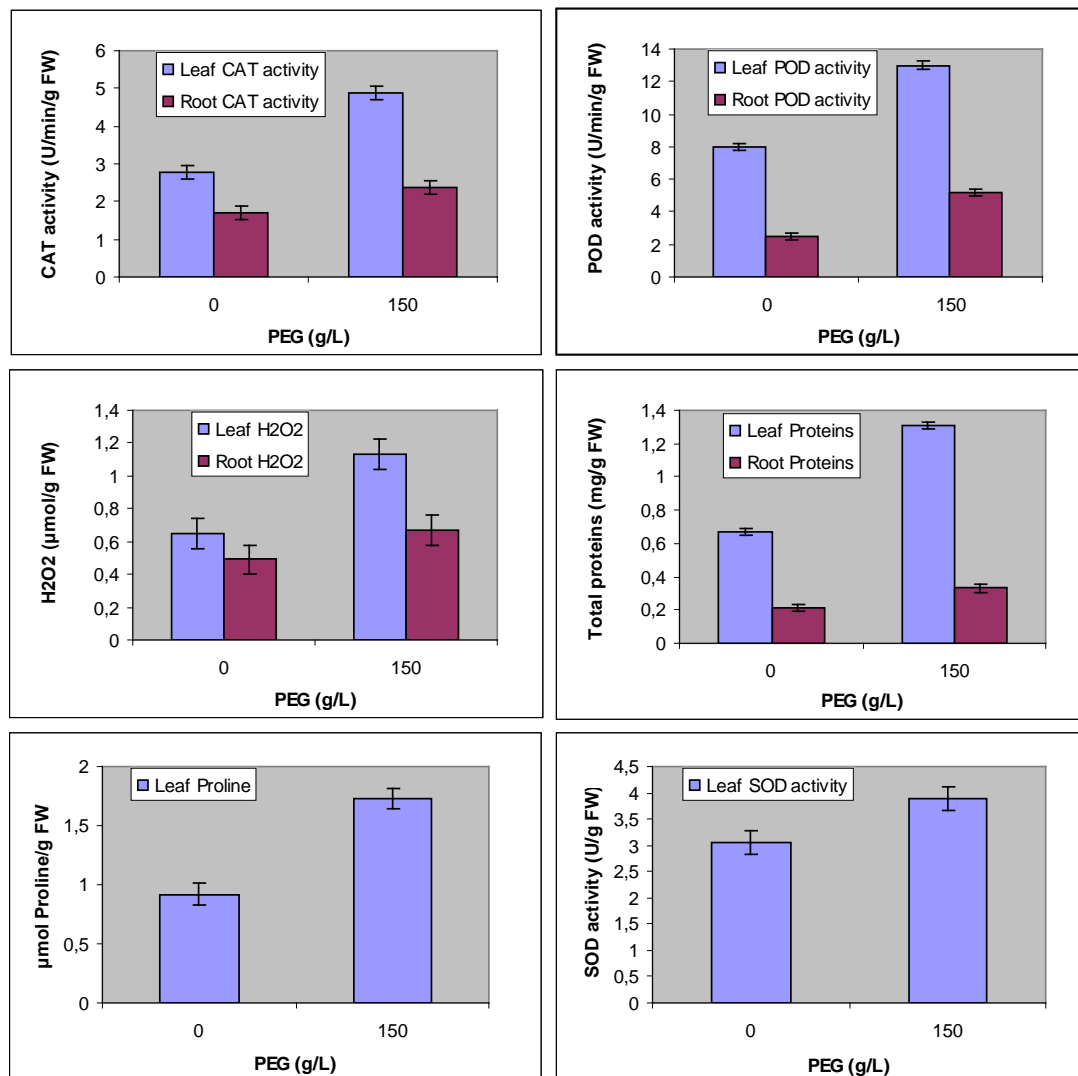
Fig. 1. The effect of salinity (NaCl concentration) on peroxidase (POD) activity in strawberry leaves and roots. Bars represent ± SE values (n = 4).



Experiment 2

Drought effect increased immediately the formation of hydrogen peroxide (H_2O_2) in leaves and roots. Reactive oxygen species (ROS) like H_2O_2 are considered as cellular indicators of stresses as well as secondary messengers actively involved in the stress response signalling pathway. It seems that H_2O_2 triggered the leaf and root antioxidant mechanism. Particularly the increase of H_2O_2 was accompanied by the induction of the key-antioxidant enzymes catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). Thus it seems that in strawberry leaves and roots SOD, CAT and POD take part in defence mechanism against oxidative stress as a plant mechanism to detoxify ROS. Drought stress was also accompanied by increasing protein content which may be explained by the production of stress related proteins which include the antioxidative enzymes. Furthermore, drought induced changes in the accumulation of proline, which is often used as an indicator of stress tolerance and this may be correlated with the ability of osmotic regulation of 'Camarosa' to tolerate water limitation. Nevertheless, more extensive measurements are needed to assess long-term drought effects.

Fig. 2. The effect of drought induced by PEG on enzymatic activities of catalase (CAT) peroxidase (POD) superoxide dismutase (SOD) and protein, proline and hydrogen peroxide (H_2O_2) content in strawberry leaves and roots. Bars represent \pm SE values (n = 6).



Conclusion

The objectives of the STSM were achieved. The results obtained revealed that:

1. Hydrogen peroxide seems to be considered as an indicator of stress as well as secondary messenger actively involved in the stress response signalling pathway in strawberries.
2. Strawberry plants under salt-stress conditions retained the phenylpropanoid and flavonoid pathways intact and functioning enabling plants to respond to oxidative stress for defence requirements (nonenzymatic antioxidative molecules involved in redox reactions).
3. Quantification of enzymatic activity under drought conditions showed that strawberry plants possess specific mechanisms to detoxify ROS which include activation and cooperation of antioxidant enzymes such as SOD, CAT and POD.
4. Proline may be an indicator of stress-tolerance in strawberry and may be correlated with osmotic regulation and protection.

In the face of a global scarcity of water resources and the increased salinization of soil and water, abiotic stress is already a limiting factor in plant growth. Moreover, stress-induced production of ROS is another aspect of environmental stress in plants. In view of the above, alleviation of oxidative damage by the use of different antioxidants and ROS scavengers (high constitutive or induced levels), can enhance strawberry plant resistance to salt and drought (Vinocur and Altman, 2005). Although the process of 'priming' or 'hardening', which involves prior exposure to a biotic or an abiotic stress factor making a plant more resistant to future exposure, has been known for years, it has been appreciated just recently (Beckers and Conrath, 2007). For example a 'hardening' technique (water shortage, salinity treatment, hardening-inducing compounds) in strawberry plants may become an important tool in strawberry stress tolerance, without heavily affecting fitness.

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Acknowledgements

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Grant Request for STSM of Dr. Damianos Neocleous

Budget Request: Year-2009

Travel 200 Euro

Subsistence (hotel/meals) 2300 Euro

Total 2500 Euro