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The effects of environmental factors on product quality of vegetable crops

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Abbreviations

AA	Acetaldehyde
ADH	Alcohol DeHydrogenase
BLSS	Biorigenerative Life Support Systems
CG	Control Greenhouse
CLC	Crew Latent Condensate
DM	Dry Matter
DW	Dry Weight
EVA	Ethylene-Vinyl Acetate
FA	Formaldehyde
Fm	Maximal fluorescence from dark adapted leaf
Fm'	Maximal fluorescence from light adapted leaf
Fo	Fluorescence emission from dark adapted leaf
Fo'	Fluorescence emission from light adapted leaf
Fv	Variable fluorescence from dark adapted leaf
Fv/Fm	Maximum quantum efficiency of PSII photochemistry
Fv'	Maximal fluorescence from light adapted leaf
FW	Fresh Weight
ISS	Intenational Space Station
MEI	Methanol, Ethanol, Isopropanol
NIR	Near Infra-Red
NSC	Non Structural Carbohydrate
oxVOC	Oxidised Volatile Organic Compound
PAR	Photosynthetic Active Radiation
PG	Photovoltaic Greenhouse
PPFD	Photosynthetically Active Photon Flux Density
PVB	Polyvinyl Butyral
PVC	Poly(methyl methacrylate)
PVP	Photovoltaic Panel
SLDW	Specific Leaf Dry Weight
UV	Ultra Violet
VOC	Volatile Organic Compound

Introduction

Aim of the thesis

European stakeholders agree on the need to improve the transfer of innovation from academia to industry in order to have both economy and society benefit from that. Such improvement is a challenge that governments are trying to address by financing tightly focused projects whom seek to put into close collaboration public research bodies and private industries. For the academia this would represent a tremendous opportunity and a commitment that would also imply education of young researchers, post docs and PhD students to operate in emerging fields to catch opportunities of research funding and employment. The work done in this Thesis was part of different funded projects carried out in tight collaboration with industrial partners (both small-medium enterprises and large companies) along with other academia collaborators. The knowledge in "role of plant-environment interaction on quality of agricultural products for the market" enhanced by this Thesis would help industrial partners meet high quality standards in various fields of application, including horticulture, space biology, nutraceutical and biorefinery. Methodology, species and goals of singular projects may be slightly different according to the aim of the experiment drafted in accordance with Italian and foreign companies involved in the activity. This contributed to broaden the Author's knowledge of physiological and biochemical traits that shape the final quality of product on the market. Moreover, it was of great benefit to learn and experience broad and tight collaborations with industrial partners.

Food quality and food security

Food quality and food security have important implications on the human diet across the world. "Food is defined as any substance that people can eat and drink to maintain life and growth" while food security is "adequate access to food for all people at all times for an active, healthy life" (*1*). Nevertheless, the quality of food is more difficult to define. Quality is often associated to a subjective interpretation of one or more characters of a given thing. When it comes to food, standards have to be set and respected, in many cases food quality requisites are defined by law. The term food quality embraces several properties like: aroma, appearance, chemical constituents, taste, nutritional values etc (*2*). Here, it is referred to quality of fruits and vegetables as the concentration of compounds that affect their nutritional, organoleptic and nutraceutical (DeFelice in 1989 cited by

Kalra 2003 (*3*)) value. Food quality and food security are among the most important issues on the international agenda of many governments, but policymaking systems struggle to produce effective broad regulations and actions to make people satisfy their daily energy intake. During the years 2012-2014 approximately 805 million people suffered from chronic hunger and could not conduct an active life due to food intake deficiencies. The end of 2015 is the target date for achieving the Millennium Development Goals (MDG) (*4*). The involvement of all stakeholders worldwide could not be sufficient for defining the strategies needed to achieve such import goal. In my believe, new insights regarding the role of environmental factors on determining fruit and vegetable quality can be viewed as a modern and effective way to ameliorate the quality of food for a better nutrition under many economic and social situations.

Plants: a valid source of nutraceutical compounds and high quality biomass

Higher plants represent a valid source of food for human diet providing both macro and micronutrients, including functional molecules. Plants are autotrophic organisms that use light, water and inorganic carbon for the synthesis of carbohydrates. Sucrose and starch that can be considered direct products of photosynthesis are used by plants as fundamental forms of energy for transport and accumulation, respectively. By strict metabolic definition, the first photosynthetic products are triose phosphates that are used to synthesise sucrose in the cytoplasm and primary starch in the chloroplast. Sucrose can be exported to all heterotrophic tissues of the plant, where it provides energy and reduced carbon to all cellular functions, including growth, respiration, and accumulation of secondary starch. Plant carbohydrates, particularly starch, have represented the most reliable source of energy for people all over the world and throughout the history. In adults, the contribution of carbohydrates to the total energy intake ranges between 36.8 and 51.0% in men and between 37.7 and 51.8% in women (*5*). Cereals have contributed to human diet for millennia representing one of the most reliable source of carbohydrates. Wheat, rice and maize are the most cultivated cereals all over the world and they account for the 53% of the whole cultivated land (*6*). In Europe, cereals account for 25% of the EU's crop production value and a third of the total agricultural land (*7*). Carbohydrates also contribute to taste giving sweetness to food (*8*).

Additionally, carbohydrates of cereals and vegetables contribute as main source of fibres in the human diet. Fibres encompasses many macromolecules that have structural as well as storage function in plants like polysaccharides oligosaccharides (*9*). Although fibres

have been considered for many years not relevant in human nutrition for their low energy value, they have gained in the last decades more and more importance for their contribute to the proper functioning of the gut (*10*).

Nutritive properties of plant foods also come from amino acids and fat. Protein amino acids join together in order to form proteins and play a role of intermediates in the metabolism of both plants and animals. Proteins can be enzymatically broken down into amino acids during the digestion and absorbed from the intestine. Under nutritional point of view, some amino acids are considered essential because human metabolism either lacks of specific pathways for their synthesis or the synthesis, is so slow to meet the organism daily need (*11*). Therefore, different sources of proteins should be taken into account in order to avoid amino acid deficiencies. Moreover, moderately high protein diet has beneficial effects on the cardiovascular system (*12*). Protein consumption account for the 13-19% of the total energy intake in European adult men and women (*5*). Cereals are the most important source of plant proteins in Europe providing 42-69% of the total protein intake for men and 35-61% for women; Italy is among the European countries where cereals represent the major source of proteins (*13*).

Fats are mainly used by plants as carbon and energy storage. Fats along with oils (respectively solid and liquid at room temperature) belong to a general class of lipids. They are prevalently accumulated in seeds (in order to have seedlings meeting the energy requirement for germination) and fruits. Fats and oils represent the most reduced form of carbon. In fact, the complete oxidation of a g of either oil or fat contains about 40kJ and it can produce far more ATP than a g of starch (15.9kJ) (*14*). Fat, is a non-transportable form of energy within the plant tissue. Then, it is generally converted into sucrose for transport via gluconeogenesis. In human nutrition fat and oil intake is not just a source of energy but they enhance the assimilation of fat-soluble vitamins like Vitamin A, D, E and K (*15*) and provide necessary unsaturated fatty acids (linoleic acid and alpha-linoleic acids). New findings suggests that among lipids, fatty acids are linked to taste sensory and they could affect dietary behaviour: low oral sensitivity to fatty acids may contribute to obesity (*8*).

Nevertheless, they do not contribute to the human nutrition only for their content of macronutrients and fibre. Many plant metabolites in fact have positive effects on human health while other may pose a threat, others are essential like vitamins or provitamins (a

compound that could be converted into vitamins by animal body). Ascorbic acid or Vitamin C is probably the most known organic acid with antioxidant properties that plants synthetize in three differ forms: ascorbate, monodehydroascorbate and dehydroascorbate. Since Vitamin C is an electron donor, it can scavenge ROS (Reactive Oxigen Species). Unlike many animals, humans cannot synthetize Vitamin C, therefore they have to rely on fruits and vegetable for maintaining healthy concentration of such molecules. A severe limitation of Vitamin C nutritional intake causes scurvy, a potential deadly illness. The total pool of Vitamin C in a healthy adult is approximately 1500 mg, scurvy may occur at level as low as 350 mg, a value that can be reached if Vitamin C is not taken in for 60-90 days continuously (*16*). Although a daily intake of 10 mg is enough to guarantee the minimum Vitamin C supply an optimal daily intake in several times higher for many human categories. According to the World Health Organization a daily consumption of fruits and vegetables should be higher than 400 g per capita (*17*). Usually, organic acids are not abundant in green vegetables but they contribute to taste properties giving tartness to food and affecting the perception of sweetness. Among them, malic acid and citric acid are the most abundant organic acids in green vegetables (*18*). Sugar/acid ratio is often used for characterizing the taste and the degree of ripeness of fruits (*19*). Vitamin E, A, K, D are also synthetized by plants and strengthen the qualitative properties of plant food along with polyphenols, glucosinolates and other antioxidant agents like lycopene that is particularly abundant in tomato fruits.

Several other metabolites that are not directly involved in either growth or development of the plant are normally synthetized and stored in different organs for accessory purposes: they are known as secondary metabolites. Secondary metabolites are organic molecules that can be clustered into four major classes: polyphenols and polyphenolic compounds, terpenoids, alkaloids and sulphur-containing compounds (*18*). Some molecules from the second metabolism are known to be species-specific while other have broader contribution; in both cases, they have a great variety of roles in plants. They exert defence against pests and pathogens, protection from UV and other abiotic stress factors as osmotic stresses, allelopathic interaction with other plants (*20*). Alkaloids for example are molecules with high biological activity. Many flowering plants accumulate alkaloids for their repellent role against vertebrate and invertebrate (*21*). However, not all secondary metabolites have such effect. For instance, oxalic acid is considered negative for human diet since it can increase the risk of kidney stones (*22*); it is found as soluble

acid or insoluble salt at different concentrations according to the plant species, environmental factors, ages and agricultural practices (*23*).

Primary metabolites and secondary metabolites are qualitatively and quantitatively regulated by plants in order to cope with external environmental conditions. In fact, growth factors as light, temperature, soil conditions as mineral nutrition and salinity, CO_2 availability can affect specific metabolic pathways and in turn plant food quality. Therefore, further investigations on the role of environmental factors would help to increase desired primary and secondary metabolites whilst reduce accumulation of undesired ones in order to enhance the overall quality of human diet.

Environmental factors and their effect on quality of plant food

Unlike animals, plants are sessile organisms that cannot move elsewhere if conditions change over time. They have to adapt or acclimate as much as possible to the surrounding environment in order to survive and reproduce, eventually. Precisely, adaptation involves the heredity of genetic traits gained throughout the previous generations while acclimation is referred to the physiological response of the plant to environmental conditions during the lifetime (*24*). Adaptation is exhibited in different ways: growing habitus, thickness of leaves, presence of thricomes, shape of leaves, flowering period, roots structure and expansion, photosynthesis metabolism (C_3, C_4, CAM) and so on. Acclimation also involves many aspects of the plant structure and function that include, among others, capacity of the photosynthetic apparatus and functioning of gas exchanges, size and structure of leaves and other organs, accumulation of specific metabolites in response to stress factors. Both adaptation and acclimation are responsible of the quality of vegetables but the effect of acclimation can be exerted in very short period in response to changes in environmental growth conditions so that it can be used to tune the quality of horticultural products. Light is among the most influential factors that affect plant growth and development (*25*). It has played important role during the evolution of the plant kingdom making every species selected for its capacity to deal with a given intensity of light for a given photoperiod. Light is the source of energy for autotrophic plant growth, and hence it is the main driver of productivity (*26*). The photosynthetic production of energy and hence of reduced carbon is strongly dependent on light intensity which is frequently a limiting factor for plant productivity in controlled environment agriculture. Light quality can also affect plant proactivity and quality. The importance of light quality on productivity and quality in increasing with the rising relevance of controlled

agriculture on food production. Acclimation to light is often visible at naked eye for the morphological changes that the plant undergoes as etiolation, leaf number, leaf thickness, period of flowering and fruit ripening but there are many others that can not be seen if specific analysis are not performed. Changes occur at the physiological level via stomata regulation, transpiration rate and photosynthesis rate regulation. Others occur at the metabolic level with delicate adjustments of specific metabolic pathways. The plant enzyme nitrate reductase (EC 1.7.1.1), for example, is a light dependent enzyme responsible for the reduction (NADH dependent) of nitrate into nitrite. Its activity is regulated by an array of factors at both transcriptional and post-translational level and light plays an important role in its activation in terms of photoperiod and intensity of light (*27*). Low nitrate reductase activity coupled with high nitrogen supply would lead to an accumulation of nitrates in the vacuole of plant cells. Ingestion of high quantity of nitrates is thought to be detrimental for human health and because of that, farmers that produce leafy vegetables as lettuce, spinach and rocket have to be in compliance with the European regulation 1258/2011 (*28*, *29*). Exposure to different light intensities can influence the yield and quality over the course of time; usually, low light intensity is correlated to lower production of metabolites as ascorbic acid in green vegetables and lower β-carotene content in tomato (*30*). Low light conditions can affect negatively the marketable quality of vegetables as well (*31*). On the other hand, excessive light intensities can have detrimental effects on yield and quality. The development of illness like sunscald can jeopardize the production of tomatoes, bell peppers and eggplants (*32*). Moreover, light affects the temperature particularly in greenhouses. In close environment cultivation, temperature control is fundamental for avoiding important losses. Quality and taste of tomato fruits are lower when plants are cultivated in suboptimal temperatures: fruits are usually less juicy, less aromatic, less acid and less keeping quality (*33*). Colour of tomato is also affected by temperature: colour is attributed to synthesis and accumulation of lycopene and carotenoids in the fruits. They both are thought to be dependent to temperature (*32*).

The plant root system is partially selective for external compounds that could potentially interact with the plant metabolism. Since the delicate equilibria within the plant metabolism, roots exert the first barrier for external compounds in the uptake process. Mineral nutrition is largely mediated by root absorption, metabolism and translocation. The same mechanisms is invoked when plants have to deal with organic pollutants that

can easily occur in the substrate. Unbalanced mineral nutrition and the occurrence of inorganic as well as organic pollutants in the substrate are an environmental factor that can trigger undesired accumulations of toxic compounds in the plant tissues which, in turn, would affect negatively the overall quality of edible parts of the plants. In many regions of the world water and soils are polluted, inadequate horticulture practices could lead to the production vegetables containing pollutants putting under threat a large fraction of the population for unsafe nutrition. Because of possible occurrence of inorganic and organic pollutants in agricultural lands, horticultural practices should be wisely considered in case of polluted soils in order to avoid accumulation of toxic compounds in plant food (*34*). Polluted soils are normally unlawful and not used for food production in many countries. A possible strategy to overcome this problem is represented by soilless horticultural practices.

The constant increasing of soilless cropping systems in the world is due to the possibility to modulate carefully the quantity and the quality of the nutrients available for plants. Water use efficiency, soil-borne diseases, fertilizer savings and avoidance of pollutants in plant food are only few of the many advantages of such cropping systems (*35*). Hydroponic systems are very versatile and there are many possible applications, even in extra-terrestrial environments.

Still, the effect of the substrate on vegetables is not fully understood especially because different species may have different response to the same edaphic factor. Non-inert substrates affect plant uptake of nutrients at the level of ion exchange and if pH and salinity are not kept under control, nutritional deficiencies in plants may arise. Salinity is one of the major issue farmers have to deal with for cropping. Increasing in soil salinity is usually associated with a decreasing in productivity. This is due to toxic ions effects on plant metabolism, to osmotic disturbance to main physiological functions like osmotic balance at the cellular and sub-cellular level, stomatal control, water uptake, photosynthesis, growth, and protein synthesis. However, moderate increasing of the salinity could enhance the overall quality of vegetables; moderate salinity decreases nitrate content in leaves of rocket (*28*), increases polyphenol content and antioxidant activity in leaves of cardoon (*Cynara cardunculus* L.) (*36*) and fosters lycopene levels in tomato fruits (*37*).

Plants all over the world have to deal with broader environmental changes that would affect food production, quality and safety (*38*). The constant increasing in the environmental concentration of CO_2 is promoting the greenhouse effect, which in turn affects the global temperature. Small variations in the temperature would compromise the stability of glacial caps upsetting many delicate equilibria in nature with catastrophic events (*39*). Ironically, plants could benefit from such increasing in the atmospheric CO_2 particularly in many relevant agricultural species. At ambient CO_2 partial pressure, the photosynthesis, particularly in C_3 species, increases proportionally to the increasing in CO_2 availability. This in turns causes an increasing of carbon gain and, ultimately, of productivity unless other factors like light intensity, become limiting. Literature is plenty of reports indicating that yield and quality of vegetables can be fostered by increasing CO_2 availability to plants. Hence, growers have tried to exploit this new findings as first benefits may come from higher carbohydrate production and storage in plant tissues, that would turn the biomass produced into a more suitable substrate for for industrial processes. In this specific case, the production of biomass is not strictly related to quantity of food produced but it has an additional value as substrate for the industry. Sugars can be converted into alcohols and gasses via fermentation by microorganisms. Using plant sugars for biofuel production could represent a valid alternative to fossil fuel because the renewability and availability in large scale (*40*). Potentially, all plants are suitable for fermentation but when it comes to efficiency, it is not true. In fact, what is converted into alcohol is sugars therefore, low availability of free sugars in proportion to the total bulk digested decreases the efficiency of the process. Hence, increasing the content of fermentable sugars in biomass, by fostering the synthesis and accumulation of NSC (non-structural carbohydrates), would in turn increase the quality of the substrate employed in industrial transformation.

The control of environmental factors for high quality food on earth and in space

In many agricultural systems plant proactivity is limited by suboptimal environmental factors, and this problem will worsen in the future due to climate change. Controlling the environmental factors for a significant increase in the efficiency of staple food cultivation is not as easy, breeding and fertilizations are the most effective tools for driving such amelioration. But it is feasible for a large number of high value crops. Many vegetables and fruits are already produced in partially controlled environments systems such as tunnels and greenhouses with high yield per unit of land exploited and an intensification

of the environmental control can contribute to increasing food quality and safety. The cultivation in fully controlled environments would be an unavoidable prerogative in manned space missions. Plant growing in space should be set in confined volumes where exchanges of mass and energy must be tightly controlled. The contribution of plant growing systems to the crew nutrition and life support (production of O_2, fixation of CO_2 purification of water and phytodepuration) is fundamental for future missions (*41–43*). It will be just demonstrative in the International Space Station (ISS) according to the NASA plan to grow salad in the Veggie module (*44*). In manned long duration missions and in planetary settlements plants should be increasingly used for life support as the distance and duration of the mission increase. In case of large scale cultivation in extra-terrestrial human settlement, higher plants would play a pivotal role in the regeneration of oxygen, food production, waste recycling and preservation of astronauts' wellbeing (*45*). In such type of missions, conceivably, plant should supply the crew member with staple food and with fast growing high quality vegetables for best possible nutrition and should guarantee the proper supply of, carbohydrates, proteins, fat fibres, vitamins antioxidant compounds, and minerals. Besides, in space applications the area designable for cropping is not infinite. Then, in Bioregenerative Life Support Systems (BLSS) high standards of productivity and efficiency must be met. Vegetables and fruits cultivation productivity, efficiency and safety in space must can be increased using specific varieties and breeding can contribute to the goal, but a proper understanding and management of the effects of environmental conditions on the nutraceutical properties of plant food is fundamental.

General methods

Plant physiology: fluorescence and gas exchange measurements

Plant physiology assessment was conducted with non-destructive methods that implied the use of a Li-Cor 6400xt equipped with a leaf chamber fluorometer couvette (mod. LCF-1005) for analysis of chlorophyll *a*, fluorescence in dark and light adapted leaves and gas exchanges. In all measurement sessions, leaves were left to acclimate to the cuvette conditions that were set as similar as possible to the growth conditions used for a given experiment. Furthermore, the period of the day was also taken into account while recording leaves' performances in order to avoid possible midday depression. Fluorescence on dark adapted leaves was recorded after 30 minutes of exposure to

complete dark environment; leaves were selected among the most fully expanded ones, orthogonally exposed to light and visually healthy. Fluorescence measurements provide reliable estimation of the quantum yield of PSII photochemistry. Defining Fm as the fluorescence measured as PSII reaction centres are maximally open, Fo the fluorescence measured as PSII reaction centres maximally closed and Fv the difference between Fo and Fm, a reliable value regarding the quantum yield of PSII photochemistry can be derived by the ratio Fv/Fm (*46*). The mean value of Fv/Fm ratio for non-stressed leaves of C_3 plants (as tomato, rocket, spinach and cardoon) is assumed to be 0.832±0.004 with values ranging from 0.754 in case of thin shade leaves of cotton to 0.896 in case of thick leaves of Eucalyptus (*47*).

Growth analysis

Growth analysis permits to better understand if changes in environmental factors have affected the phenotype. Morphological changes may occur when plants acclimated to different conditions; for example, it has been known since Roman times that there is proportionality between the upper and below-ground part of the plant in response to environmental factors (*48*). The capacity to interact with the environment is dictated by genetic but the analysis of the growth would help top figure out how plants acclimate to different environmental conditions. Growth analysis may change slightly according to the purpose of the experiment. Routinely, the plant was divided in roots and leaves or scion at the collect level. Fresh weight was recorded, then samples were freeze-dried until constant weight for dry matter content assessment. In care of fruits, they underwent the same procedure. In case of leafy vegetable the total leaf area, Specific Leaf Dry Weight (SLDW) and number of leaves were also recorded. The specific leaf dry weight (dry weight/leaf area) was measured on leaf discs, cut from the lamina with sharp cork borers of known area. Dry weight was obtained by freeze drying discs to constant weight.

Qualitative analysis

Plant tissues were finely grounded in liquid nitrogen with chilled mortar and pestle for analysis of: non-structural carbohydrates (glucose, fructose, sucrose and starch), chlorophyll and carotenoid content, soluble and non-soluble proteins, nitrate and malic acid. Approximately 100 mg of ground tissues were usually extracted in glass-glass

homogenizer with 1.2 ml of buffer solution made of 100 mM of Hepes (pH 7.1) and 10mM $MgCl_2$. An aliquot of 200 µl was extracted in 800 µl of 100% ethanol, at 80 °C for 45 min and centrifuged at 12000 g for 5 min for non-structural carbohydrates assessment. Soluble sugars (glucose, fructose and sucrose) were measured in the supernatant, while starch in the pellet. The pellet, containing starch, was re-suspended and washed three times with 50 mM NaAcetate buffer (pH 4.5), autoclaved at 120 °C for 45 min in 1 ml of the same buffer, and finally incubated at 50 °C for 1 h with amyloglucosidase and α-amilase to hydrolyze starch to glucose. Starch content is reported as glucose equivalent. Soluble and non-soluble carbohydrates in biomass samples were analyzed by high-performance anion exchange chromatography, with pulsed amperometric detection (HPAEC-PAD) (Thermo Scientific™ Dionex™ ICS-5000, Sunnyvale, CA U.S.A.), consisting of a isocratic pump, a pulsed amperometric detector, an injection valve with a 5 µl injection loop, and an analytical CarboPac SA10 column (4 mm x 250 mm) with the guard column. The detection cell contained a gold working electrode (1.0 mm in diameter) and an Ag/AgCl reference electrode. Pulsed amperometric detection was carried out with the following waveform: E1= +0.10 V (t1=0.4 sec), E2= -2.00 V (t1=0.01 sec), E3= +0.60 V (t=0.01 s), E4 = -0.10 V (t=0.06 s). The integration range began at 0.2 s and ended at 0.4 s. The electrical signal was integrated in nanocoulomb (nC). Runs were carried out at 45 °C. NaOH (1 mmol l^{-1}) was used as mobile phase at a flow rate 1.5 ml min^{-1} with a post-column addiction of more concentrated hydroxide (300 mmol l^{-1}). Post column addition was made using a second pump, at a flow rate 0.5 ml min^{-1}. The eluents and the carbohydrate standard solutions were prepared using HPLC grade reagents (Sigma, Steinheim, Germany). The IC method required minimum sample preparation and the analytes could be separated without interferences.

From the initial 1.2ml homogenized sample, a second aliquot of 200 µl was used for soluble protein content assessment that was carried out according to the principle of protein-dye binding (*49*).

A third aliquot of 500 µl was further extracted in 500µl of buffer solution made of 100 mM of Hepes (pH 7.1), 10 mM $MgCl_2$ and Triton 0.02 % in glass-glass homogenizer for content of proteins chlorophylls and carotenoids. Total protein content was assessed according to protein-dye binding (*49*). An aliquot of 100µl was further extracted in 900µl of 100% acetone for chlorophyll and carotenoid measurements that were accomplished

via spectrophotometric assay by measuring the absorbance at 470, 647 and 664 nm wavelengths with a UV-V spectrophotometer (6715 UV/Vis Spectrophotometer Jenway Bibby Scientific -UK). The extinction coefficients used for the determination of total chlorophyll and carotenoids were those described by Lichtenthaler and Wellburn (1983).

Malic acid content was determined extracting plant tissues as described for soluble sugars extraction. Quantification was performed via enzyme-coupled spectrophotometric method, as described by Famiani et al. (2009). The extraction and determination of vitamin C (ascorbic acid) was performed using the protocol of Kampfenkel et al. (1995). Ascorbic acid content was measured from freshly harvested material, using leaf discs (about 2 cm^2 of area). Each disc was taken and immediately frozen and stored in liquid nitrogen until required. Discs were ground using a glass-glass homogenizer containing 1.5 ml of 3% (w/v) TCA, the extract was centrifuged at 12000 g for 15 min. The determination of reduced ascorbate (AsA), oxidized ascorbate (dehydroascorbate, DAsA) and total ascorbate (AsA + DAsA), in the supernatant was made using a spectrophotometric colorimetric assay (Kampfenkel et al., 1995), by measuring the absorbance at 525 nm. DAsA was calculated as the difference between total ascorbate (after reduction of DAsA with 2 mM dithiothreitol) and reduced ascorbate. The spectrophotometric measurement was taken with a 6715 UV/Vis Spectrophotometer Jenway Bibby Scientific (UK).

Content of fructans was assessed extracting approximately 25 mg of freeze-dried tissue previously ground-fined in liquid nitrogen with mortar and pestle in 1.6 ml of hot water and agitated at 95°C for 60 min then centrifuged at 3000 g for 5 min. A milliliter of surnatant was filtered in DOWEX resin, rinsed 3 times with an ml of water and collected in 5 ml vials. An aliquot was filtered and injected in a ionic chromatographer DIONEX equipped with CarboPac PA20 for glucose and fructose determination. A further aliquot was hydrolyzed with HCL 150 mM as final concentration at 95°C for 2 hours then filtered and injected. Total content of fructans was estimated as difference in glucose and fructose between non-hydrolized and hydrolyzed solutions (*53*).

The concentration of nitrate was determined on freeze-dried material ground to a fine powder in a mortar and pestle. About 10 mg of the powder was extracted in 2 ml of water at 70°C for 40 min (*54*). After centrifugation at 12000 g for 5 min, the supernatant was analyzed through a Dionex ICS 5000, separated on a anion-exchange Dionex Ion Pac

As11-HC 4 µm, analytical column (250 x 4 mm) connected to a Dionex Ion Pac As11-HC 4 µm guard column (50 x 4 mm), using a suppressed conductivity detection. Eluents for the mobile phase and the flow rate were chosen to give the best resolution of the nitrate and the different organic acids. Alternatively the determination of nitrate was carried out by a spectrophotometric assay as in Beutler et al.1986 (*54*).

Data processing and statistical analysis

Data have been processed and plotted using Microsoft Excel 2013. Statistical analysis was performed using R software ver 2.15.3 (R Core Team 2013) along with Lawstat package. Shapiro-Willk test and Levene test were run in order to assess the normality of the distribution and the equality of variances of the data, respectively. Differences between the treatments were assessed via ANOVA test, P-values with $p<0.05$ were considered significant. Tukey HSD was used for post-hoc analysis.

Transparent photovoltaic panels as shading tool in sunny areas

Introduction.

Shading is a common practice in those countries where solar radiation is high either throughout the year or for just few months. High solar radiation can damage directly photosystems II causing a decreasing in light harvesting capacity of the leaves, or indirectly as a consequence of the increasing in temperature that in turn would affect transpiration rate and efficiency of biochemical processes. In both cases, reduction of yield and quality of the final product are unavoidable. Therefore it is important to keep plants protected. A common technique is to paint greenhouse roofs in white otherwise to lay one or more layers of neutral nets on the roof in order to reduce the incident light that enters the greenhouse. Whereas shading is not enough to prevent high temperatures, other active temperature control systems can take over. By monitoring the temperature inside the greenhouse, mechanical walls can open facilitating the exchange of heat from inside to outside and fans can be activated to remove hot air more efficiently but these devices need energy for running. Energy production implies, at least in part, burning fossil fuels. This would contribute to increase the cost of food production per unit of area and global CO_2 emission. Recently, the introduction of photovoltaic panels for green energy production in agricultural systems has been proposed but results are still uncertain. Conventional photovoltaic panels are not transparent and this feature makes them very unsuitable for cropping on the same surface. A dedicated area is to be reserved and competition for land use destination may arise. However, different combinations of conventional photovoltaic modules and agricultural species have been tested. Dupraz et al. (2011) demonstrated that agrivoltaic systems are possible with durum wheat cultivation accepting lower yield (19%) but higher total land productivity if energy production is taken into account. On the contrary, if photovoltaic panels could be installed on the same surface used for cropping without reducing the productivity as well as quality of food it would be an important step forwards for green agriculture.

Tangible support could come from semi-transparent photovoltaic panels (PVPs). PVPs are handmade devices (as shown in Figure 1) made of DSSC (Dye Sensitized Solar Cells) and obtained by embedding a silver based photovoltaic matrix in two layers of Polyvinyl butyral (PVB). The size of each panel is 20 cm by 20 cm; they are flexible and selective in specific wavelengths offering the possibility to cut solar radiation in the visible spectrum (400 nm-700 nm) by 50 % (Figure 2). Half of the incident light is converted

into electricity and the rest is left pass through and reach the plant beneath. In Italy for example, 37,104 ha are destined to greenhouse horticulture (ISTAT 2015). If all roofs of greenhouses were equipped with PVPs, considering a preventive 1 % efficiency, they would provide as much energy as three nuclear power plans of 800 MW each. Such quantity of energy can be injected into the grid or used right away or both.

However, the purpose of the experiment was to test just plants' response without considering the electrical-mechanical properties of PVPs. Light quality is an important prerogative for the correct functioning of the plant physiology and the consequent quality of the food produced (*56*). Long-term exposure to different light qualities modified by semitransparent PVPs may affect the morphology of the plant (and fruits in case of fruit-bearing plants), period of development, productivity etc. The aim of the experiment was to test the feasibility to use organic transparent photovoltaic panels (PVP), as substitute of conventional shadings in greenhouse cropping.

Material and methods

Here, three of the most important agricultural species cultivated in Italy were tested under long-term exposure to modified light spectrum: tomato (*Lycopersicon esculentum* Mill var. Microtom), rocket (*Eruca sativa* M. var "coltivata") and spinach (*Spinacia oleracea* L.). The effects of PVP on tomato plants have been test in both fully controlled environment and greenhouse, whilst leafy vegetables have been tested only under growth chamber conditions.

Experiment 1. In the cabinet (Fitotron SGD170 Sanyo Gallenkamp UK) shown in Figure 3 there were installed aluminium frames to hold dummies and nets in a system 3 by 3 (Figure 3) resulting in three treatments: Control (non-shaded), Dummy (shaded with PVP) and Net (shaded with neutral net). The net was bought in a local market and adjusted in order to reach the same transmittance of dummy. The idea behind was to assess if the modification of incident light would have affected productivity and quality in shaded plants regardless the intensity. Tomato plants (*Lycopersicon esculentum* Mill var. Microtom) were grown in a conventional greenhouse from seeds provided by University, sown in plastic pots 14 cm wide by 11.5 cm high filled with soil. As flowering period started, after ninety days from sowing, some plants were positioned into the growth chamber following a 3 by 3 scheme as shown in Figure 3, at constant 28 °C, 70 % of

relative humidity, 10 hours light and 14 hours night, CO_2 concentration of 400 ppm; some others into the greenhouse prototypes. In the cabinet light was provided uniformly to all treatments but obviously, the intensity of it that could reach the leaves was different in each treatment because the shading. Approximately, control, dummy and net plants received 700, 350 and 350 μE m^{-2} s^{-1} as Photosynthetic Active Radiation (PAR) respectively. Water was supplied manually twice a day. Rocket and spinach were tested only in growth chamber. Seeds were sown in pots filled with soil and left germinate in the growth chamber. The cabinet was set at the same conditions as for tomato but 25°C at day and 20°C at night were set. Plants were harvested after 21 days from germination.

Experiment 2. Performances of tomato were also tested from August 14th 2012 to October 7th 2012 in greenhouse-like environment. Plants were grown from seeds provided by Tuscia University. Seeds germinated in a conventional greenhouse in Tuscia University's farm and they were moved into the greenhouse prototypes as flowering started, approximately 90 days after the germination. Plants grew in plastic pots 14 cm wide by 11.5 cm high filled with soil and watered twice a day through a dripping system. Greenhouse prototypes were equipped with two different roof-covering materials: one with common EVA plastic film as control and the other with PVPs Throughout the experiment, temperature ranged between 19°C and 39°C in both control greenhouse (CG) equipped with EVA plastic film, and in photovoltaic greenhouse (PG). The global solar radiation in CG and PG was 630 Wm^{-2} (74.5% of the external radiation) and 470 Wm^{-2} (52.4% of the external radiation), respectively. After a month, both fruits and plants from greenhouse prototypes and growth chamber were harvested for growth analysis and fruit quality assessment. Gas exchange measurements, growth analysis, qualitative analysis on plant edible tissues and data processing were performed as described in General methods. Additional measurements for the quantification of lycopene and nitrogen content fruits of tomato were exclusively taken for this project and performed as follow.

Lycopene. The extraction was performed according to Sadler et al. (1990) (*57*) method with few modifications. Briefly, chopped red fruits were homogenized with mortar and pestle to obtain a pulp. Then 120 ± 30 mg of pulp were extracted in 5 ml of acetone 100 % for an hour and kept protected from light to prevent lycopene degradation. Samples were occasionally hand shaken, centrifuged and eventually scanned with a spectrophotometer (Jenway UV/VIS 6715) at 505 nm (*58*).

Nitrogen. The content of nitrogen was measured according to Bradstreet (1953)(*59*), a method known as Kjeldahl.

Results and discussions

Fluorescence and gas exchange. Measurements were taken as described in the previous dedicated paragraph. Fluorescence and gas exchange measurements are non-destructive methods that were performed before harvesting. In Table 1 the efficiency of PSII is reported for three species. Some statistical differences arose. Nevertheless, the gap between control and shaded plants is minimal, too low for possible evident differences in biomass production among treatments.

Comparison of photosynthetic performances in tomato, rocket and spinach was conducted relying on A vs C_i response where A is the assimilation rate (μmol CO_2 m^{-2} s^{-1}) and C_i sub-stomatal CO_2 partial pressure (ppm). Data show (Figure 4, Figure 6 and Figure 8) that growth under the PVPs did not cause large changes in the plant photosynthetic performances. Shaded plants of spinach exhibited slightly lower performances in photosynthesis response, though. At low sub-stomatal CO_2 partial pressure (SSPP) the slope of the A-C_i response is an estimate of the Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) activity (*60*).

According to Figure 5,Figure 7 and Figure 9 the slope of the curve in all treatments is similar suggesting no influent effect of shading on photosynthetic system of three species tested. Again, RuBisCo activity in shaded plants of spinach seems a little bit lower. It can be speculated that plants did not perceive the decrease in light intensity as a factor sufficient to determine a reduction of nitrogen investment in Rubisco, the most abundant soluble protein in leaves and in the world as well. Rubisco alone accounts for 50 % of the nitrogen investment born by leaves of land plants (*61*). Rubisco activity is tightly linked to light availability for 2 main reasons: firstly, ATP and NADH productions are crucial for ribulose 1,5 bisphosphate (RuBP) synthesis; secondly, the enzyme activity is conditioned by the photosynthetically active photon flux density (PPFD) (*62*). In fact, shade plants have usually lower Rubisco content per unit of leaf area as result from shifting in the final investment of nitrogen at the cell level in favour of thylakoid membrane-bound proteins that are meant for light harvesting, instead of soluble proteins like Rubisco associated with carboxylation (*62*).

Rubisco is the primary enzyme involved in the CO_2 reductive cycle (Benson-Calvin Cycle BCC) and its decrease triggered by PPFD reduction can cause in turn a decrease in photosynthetic performances. Curves on Figure 4, Figure 6 and Figure 8 show that even the simulation rate at high substomatal CO_2 concentration (Ci) was not affected by being grown under dummy in all species. At high Ci, (and high light intensity) the assimilation rate is pushed toward its maximum efficiency. At high level of Ci, Rubisco is forced to perform at high rate and process as much CO_2 as possible at the expense of oxygenation. Consequently, the light dependent reactions involved in the regeneration processes of RuBP (the CO_2 acceptor) can limit the photosynthesis rate as they cannot longer meet the increasing demand in substrate for carboxylation by RuBP. In this condition the RuBP regeneration rate equals the carboxylation rate and *A* no longer increases proportionally to the increasing of the substomatal CO_2 partial pressure, a case named RuBP-regeneration-limited photosynthesis (*63*).

Possible limitation in the regeneration of the substrate would directly affect all the successive steps of the Calvin cycle (carboxylation and oxidation) with the consequent reduction in the synthesis of triose-phosphates and carbohydrates (starch and sucrose). The last part of the *A/Ci* response curve is thought to be depending on the amount of triose phosphates available during the Calvin Cycle. If triose phosphates become limiting then the assimilation rate will tend to a plateau state, sometimes with negative response (*64*). According to Figure 4, Figure 6 and Figure 8 the regeneration of triose phosphate in shaded plants did not limiting the assimilation rate more than it did in control plants. Hence, acclimation to low light environment did not affect negatively that part of the Calvin cycle either. In all the three critical parts that the *A/Ci* curve represents (Rubisco activity, RuBP regeneration and triose phosphate utilization), control plants didn't perform better than shaded plants as it would be expected when less light reaches the leaves and plants get acclimate to such prevailing environmental condition. However, the driving force of the Calvin cycle is represented by the reducing power of ATP and NADPH synthetized during the light-dependent reaction. Those two reducing agents are the product of the electron transportation chain (NADPH) in the zeta scheme and the activity of the protein named ATP synthase which synthetizes ATP from ADP using the proton gradient in the lumen generated by the oxidation of water. Photosystem II (PSII) is responsible of water oxidation process. In fact, when light reaches PSII either directly or via energy transfer from other pigment antenna it generates enough oxidizing power

to break down water molecules into oxygen and hydrogen namely, electrons and protons. Hence, electrons will undertake the electron transportation chain towards Photosystem I (PSI) which reduces $NADP^+$ to NADPH. On the other hand, protons will generate the electrochemical gradient in the lumen that drives transmembrane proteins like the ATP synthase. Therefore, PSII is crucial for conversion of electromagnetic energy (light) into biochemical energy and its efficiency determines the overall performance of downstream processes.

The effect of PVPs on productivity and quality of Tomato, Rocket and Spinach in grow chamber. Productivity and quality in leafy vegetables and tomato are not very different among treatments.. Both leafy vegetables reported a reduction in SLDW (

Table 2 and Table 3) as it would be expected when leaves have to adapt to lower light as already highlighted by Marrou et al. (2013) (*65*). However, spinach seems to be more sensitive to incident light reduction that the others, particularly in root development Table 3. Lower productivity in plants of tomato shaded with PVPs was statistically significant in comparison with control.

The effect of different light regime on the quality of plant food is also marginal. Minor effects on glucose content in tomato fruits were recorded, plants that received less light produced fruits less rich in glucose. On the other hand, shaded leafy vegetables had the tendency to accumulate nitrates in leaves as one would have expected being light intensity crucial for the proper reduction of nitrate into nitrite.

The effect of PVPs on productivity and quality of Tomato in greenhouse prototype. In greenhouse prototypes the control of environmental conditions was less effective that in cabinet, therefore plants had to deal with higher fluctuation of temperature and humidity. Moreover, the light was not constant throughout the day but it changed according to atmospheric conditions. The intensity of incident light was high when the sky was clear and low in case of clouds. Rainfall event and wind had also effect on the overall energy exchange between the inside and outside. Due to these atmospheric agents, stomatal regulation was a pivotal activity in plant. In fact, the difference in the partial pressure of substomatal chamber and external environment drives the flux of water outwards the leaf

and the CO_2 flux inwards. This affects the availability of inorganic carbon for biomass production. However, transpiration is also regulated by several other factors either directly or indirectly like water availability in the soil (*66*) as well as quality and intensity of light (*67*).

Gas exchange measurements were out in field using a transparent cuvette in order to maintain the same source of light. Because the different coverage material of the two prototypes, plants acclimated to different quantity of light: CG plants were exposed to 600 $\mu E\ m^{-2}\ s^{-1}$ while PG plants were exposed to 400 $\mu E\ m^{-2}\ s^{-1}$. However, gas exchange measurements did not highlight any difference in photosynthesis performances in tomato plants of the two treatments. The total capacity of carbon reduction is the same Figure 8 as well as the Rubisco activity Figure 9.

Although the physiology of tomato plants was not affected by the two different light qualities (full spectrum in CG and modified spectrum in PG), significant reduction in carbohydrate content was evident in pants grown in PG (Table 11). Reduction of soluble carbohydrates, particularly glucose and fructose, was probably due to the lower availability of incident light. Lower non-structural carbohydrate content is usually associated with lower quality (*68*). Total lycopene content was not affected by PVPs (Table 12).

Conclusions

The aim of this work was to test the hypothesis that flexible Organic PVPs s can be functionally integrated in greenhouses to contemporarily shade crops and produce renewable energy for the farm needs. To test this hypothesis, tomato, rocket and spinach were grown under photovoltaic dummies with 50 % absorbance in the visible spectrum. The effects of such conditions were assessed for acclimation of the photosynthetic apparatus, quantity and quality of the product in comparison with neutral net shading to the same extent and full radiation controls.

It is recognized that plants are not particularly efficient in transforming the full solar spectrum into organic carbon chemical bounds (*69*). The first reason is that plants only collect and use a limited fraction of the sun spectrum for photosynthesis, approximately 50 % of the total energy available. Additionally, there is an intrinsic and operative inefficiency of the carboxylating enzyme Rubisco, on energy costs of mantainance (respiration) of the living organism, on limitation in sink capacity of the organism. All of

them are affected by environmental factors like the duration of the photoperiod, the partial pressure of CO_2, O_2 and water vapour in the atmosphere, temperature regime, water availability at the root system, nutritional limitation, pest and diseases. Under controlled environment, the efficiency of photosynthesis can be pushed towards its potential maximum by an array of solutions, including an increase of the CO_2 partial pressure, setting of optimal temperature regimes water and light conditions, an appropriate mineral nutrition and the avoidance of pest and diseases. Controlling the crop growing environment is possible in greenhouse systems but it requires energy. Such energy can be provided by PVPs during the day. Furthermore, results show that PVPs are a valid substitute of neutral nets conventionally used for shading in the Mediterranean region. Differences among treatments in both fully controlled environment and greenhouse prototypes, although statistically significant, are not too strong to dismiss this new technology. Agriculture may benefits in terms of energy and quality in future if PVPs are used extensively for greenhouse and tunnel application.

Future prospective

Possible application on large scale is the goal for such kind of technology. So far, costs of production are not competitive with conventional photovoltaic panels but the flexibility of the substrate where the photovoltaic matrix is sprayed, would leave open the possibility to realize an industrial system capable to produce PVPs as a continuous belt-like product. This process of production is known as roll-to-roll and it would permit the installation on undefined surfaces: the roll can be cut according to the size of the greenhouse and refold when not needed for the next season. Moreover, flexible photovoltaic panels could be adapted to several architectural shapes like tunnels in open field expanding the possible substrates suitable for energy production. Apart from mechanical properties, optical features can be adjusted according to needs. For instance, the quantity of incident light harvested for electricity production can be either increased or decreased during the production to accomplish the task as better as possible. Light intensity regulation can be related to the latitude (the closer to the equator, the less transparent PVPs will be) or to the species cultivated: heliophilic species against shade tolerant ones. Further optical adaptation is related to the quality of light. The spectrum of light intercepted by PVPs can be narrowed or broadened intercepting UV spectrum and NIR.

In conclusion, organic semi-transparent photovoltaic panels fit the greenhouse agriculture needs, they can produce green energy and offer valid protection against high solar

radiation. Investments on such technology should be encouraged for the best and greenest agriculture possible.

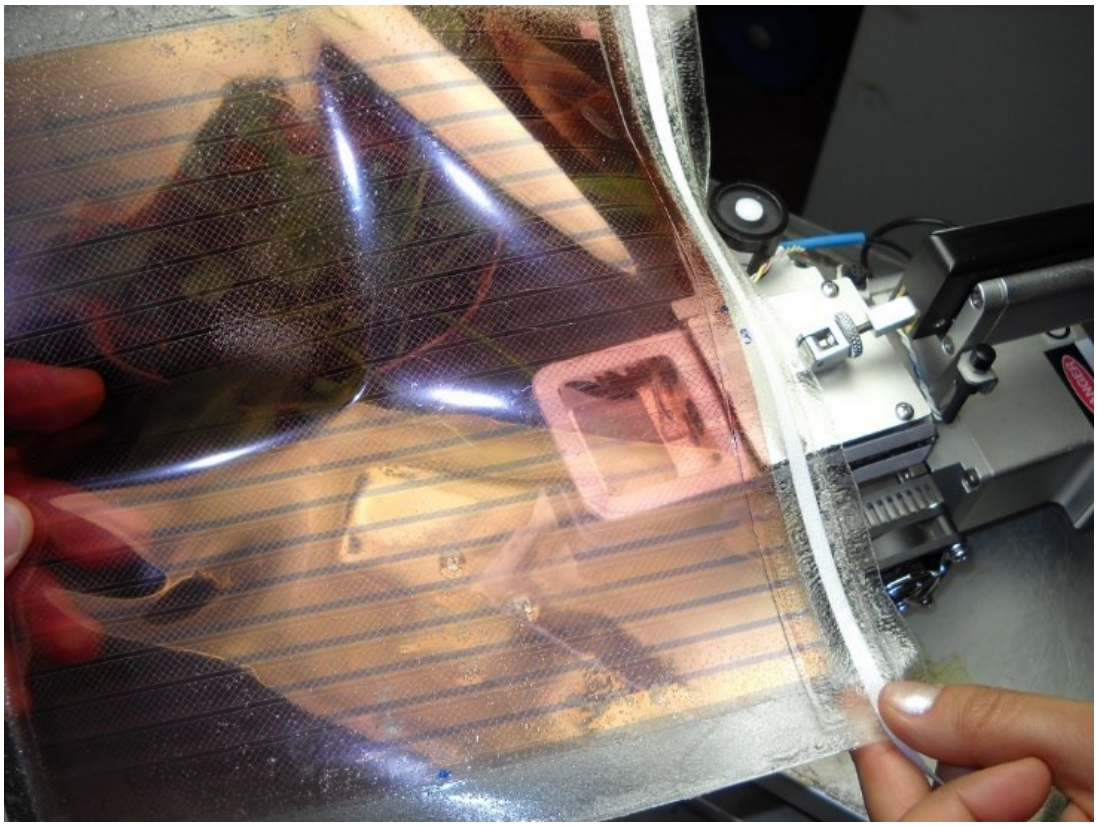

Figure 1. Semi-transparent photovoltaic panel (PVP)

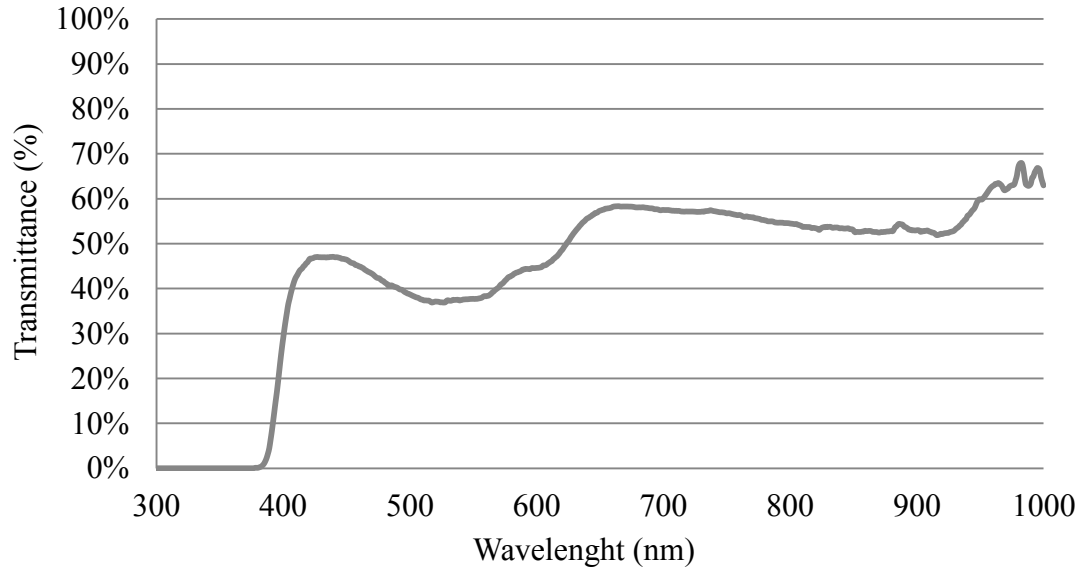

Figure 2. Transmittance of a PVP

Table and figures of plants cultivated in fully controlled environment: Experiment 1

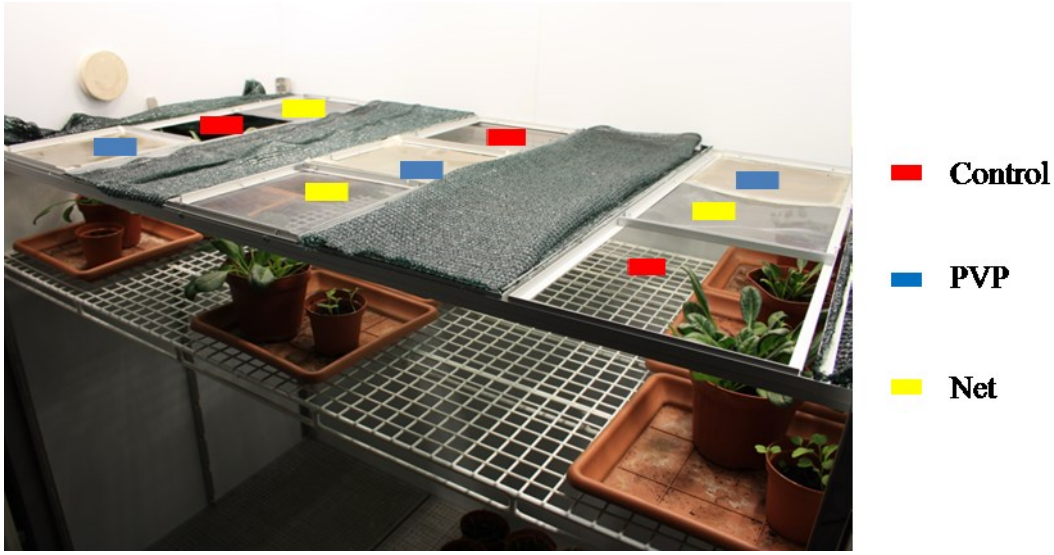

Figure 3. Fully controlled environment. Growth chanber at the Ibaf institute of CNR

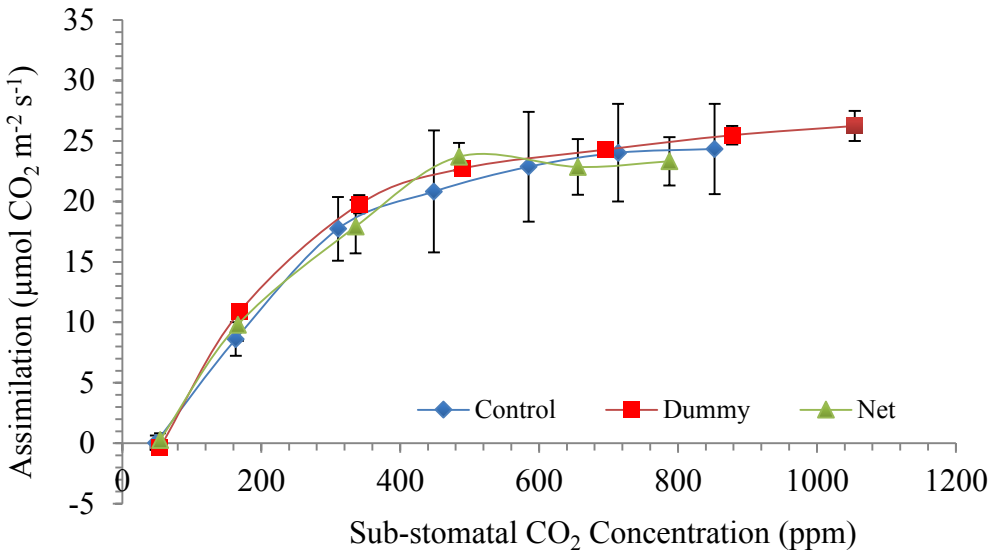

Figure 4. Assimilation vs Sub-stomatal CO_2 concentration in leaves of rocket grown in fully controlled environment under PVPs

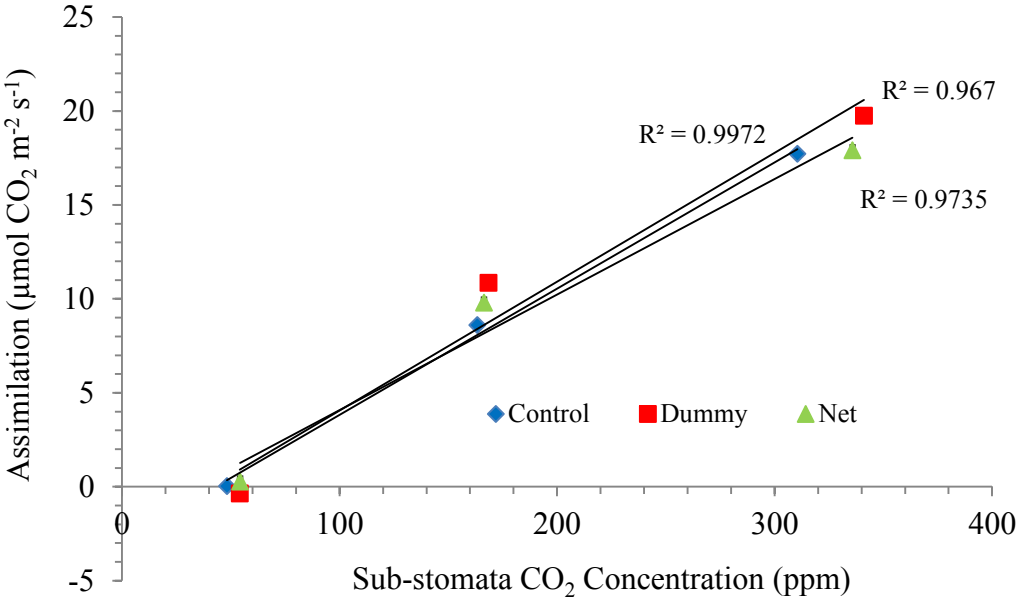

Figure 5. Rubisco activity related to the slope of the curve in leaves of rocket grown in fully controlled environment under PVPs

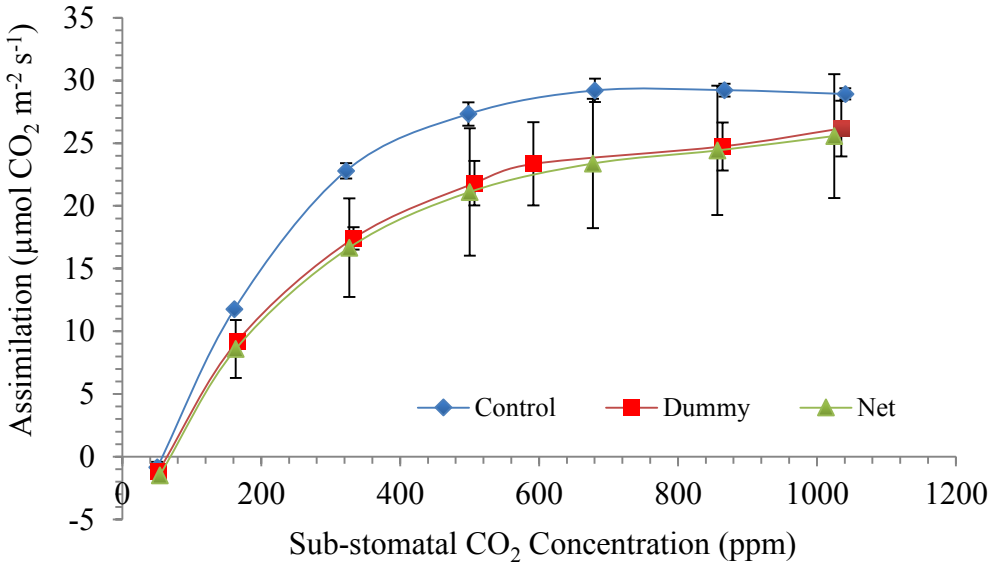

Figure 6. Assimilation vs Sub-stomatal CO_2 concentration in leaves of spinach grown in fully controlled environment under PVPs

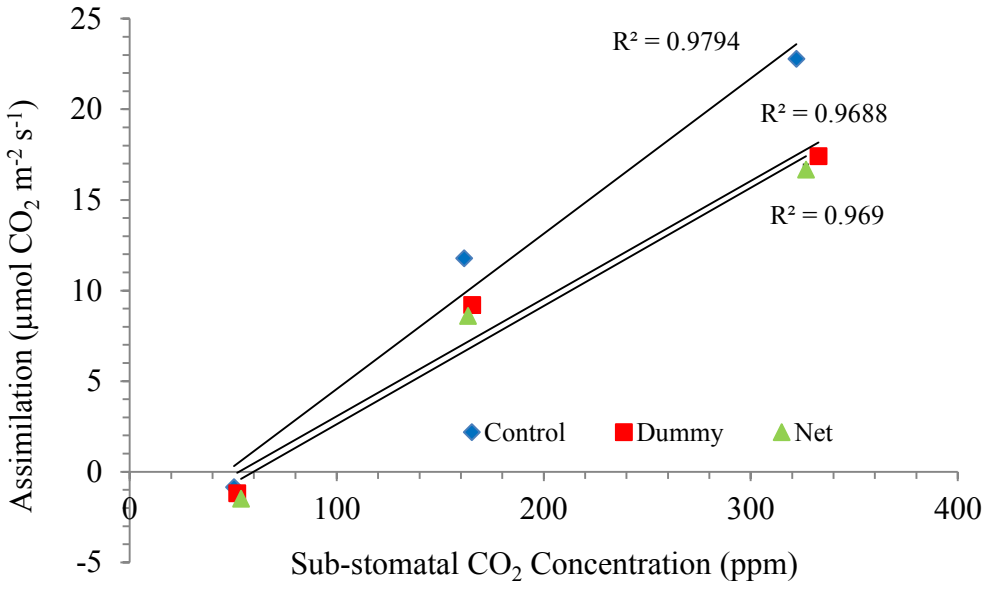

Figure 7. Rubisco activity related to the slope of the curve in leaves of spinach grown in fully controlled environment under PVPs

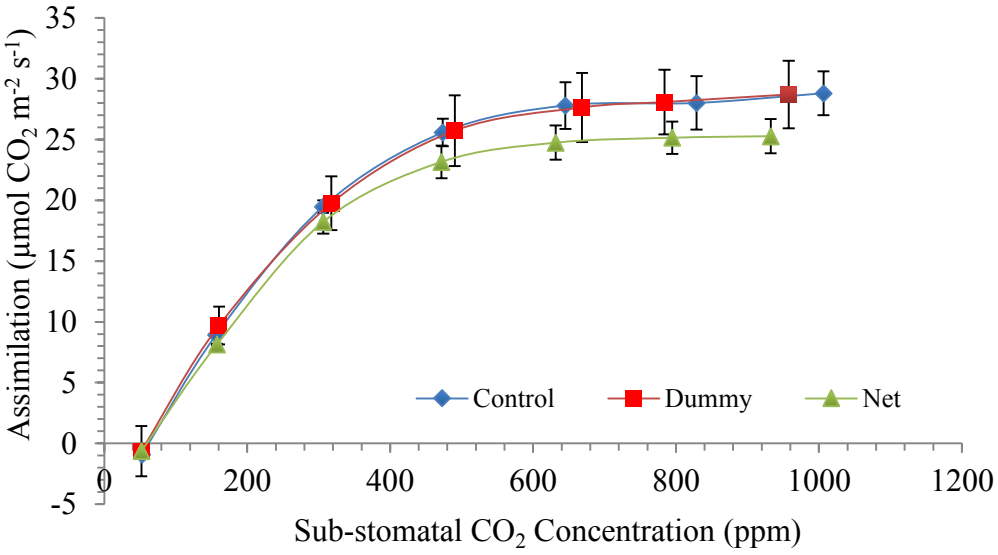

Figure 8. Assimilation vs Sub-stomatal CO_2 concentration in leaves of tomato grown in fully controlled environment under PVPs

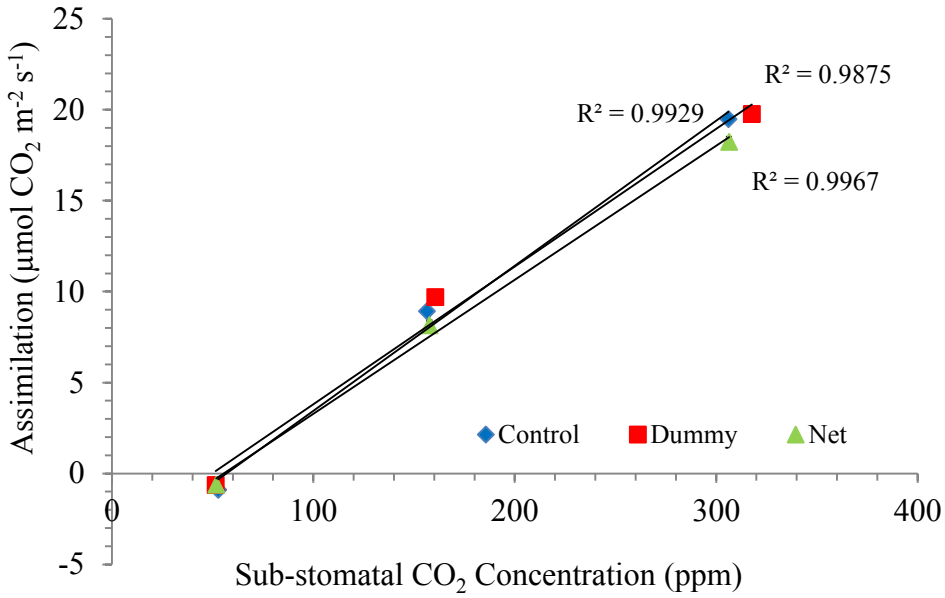

Figure 9. Rubisco activity related to the slope of the curve in leaves of tomato grown in fully controlled environment under PVPs

Table 1. PSII efficiency determined as Fv/Fm in dark-adapted leaves of rocket, spinach and tomato grown in fully controlled environment under PVPs

Treatment	Rocket	Spinach	Tomato
Control	0.82±0.004a	0.80±0.002a	0.81±0.008
Dummy	0.81±0.0002b	0.79±0.003ab	0.81±0.001
Net	0.80±0.003b	0.79±0.001b	0.82±0.002
Significance[a]	*	*	ns

[a]*ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 2. Growth analysis and productivity of rocket grown in fully controlled environment under PVPs

Treatment	Root DW (g)	Leaf FW (g)	Leaf DW (g)	Leaf surface (cm^2)	SLDW (mg/cm^2)
Control	0.11±0.03	7.16±0.58	0.92±0.08	188.8±13.09	4.64±0.43a
Dummy	0.06±0.02	4.60±0.85	0.48±0.11	139.1±19.74	3.24±0.38ab
Net	0.05±0.03	3.31±1.57	0.35±0.19	114.6±45.08	2.75±0.48bc
Significance[a]	ns	ns	ns	ns	*

*[a]ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 3. Growth analysis and productivity of spinach grown in fully controlled environment under PVPs

Treatment	Root DW (g)	Leaf FW (g)	Leaf DW (g)	Leaf surface (cm^2)	SLDW (mg/cm^2)
Control	0.21±0.02a	6.87±0.16	0.80±0.05	135.8±7.52	5.6±0.17a
Dummy	0.06±0.03b	4.09±1.75	0.44±0.21	85.9±34.56	4.1±0.48b
Net	0.04±0.01b	3.49±1.35	0.31±0.11	77.30±28.49	4.16±0.20b
Significance[a]	**	ns	ns	ns	*

*[a]ns, *,**, Nonsignificant or significant at P < 0.05 and P<0.01, respectively.*

Table 4. Growth analysis and productivity of tomato grown in fully controlled environment under PVPs

Treatment	Root DW (g)	Shoot FW (g)	Yield (g $plant^{-1}$)	Fruit DM (%)
Control	0.45±0.09	22.2±5.78	19.21±3.18	10.64±0.51a
Dummy	0.50±0.11	18.7±1.30	18.71±8.51	8.32±0.27b
Net	0.37±0.09	18.3±0.82	17.84±2.76	9.35±1.04ab
Significance[a]	ns	ns	ns	*

*[a]ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 5. Carbohydrate content in leaves of rocket grown in fully controlled environment under PVPs

Treatment	Glucose (mg/g FW)	Fructose (mg/g FW)	Sucrose (mg/g FW)	Starch (mg/g FW)	Total Carbohydrate (mg/g FW)
Control	1.74±0.73	0.14± 0.03	0.52±0.34	6.03± 3.06	8.43± 7.06
Dummy	1.11± 0.34	0.31± 0.11	0.79± 0.22	5.84± 3.22	8.05± 3.88
Net	2.43± 0.10	0.54± 0.13	0.44± 0.06	6.94± 0.61	10.35± 0.76
Significance[a]	ns	ns	ns	ns	ns

[a]*ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 6. Carbohydrate content in leaves of spinach grown in fully controlled environment under PVPs

Treatment	Glucose (mg/g FW)	Fructose (mg/g FW)	Sucrose (mg/g FW)	Starch (mg/g FW)	Total Carbohydrate (mg/g FW)
Control	0.07± 0.01	0.03± 0.01	0.80± 0.06	0.75± 0.26	1.65± 0.31
Dummy	0.05± 0.02	0.03± 0.01	0.54± 0.09	0.32± 0.11	0.95± 0.01
Net	0.12± 0.05	0.03± 0.01	0.79+± 0.30	0.24± 0.11	1.18± 0.47
Significance[a]	ns	ns	ns	ns	ns

[a]*ns, Nonsignificant*

Table 7. Carbohydrate content in fruits of tomato grown in fully controlled environment under PVPs

Treatment	Glucose (mg/g FW)	Fructose (mg/g FW)	Sucrose (mg/g FW)	Total Carbohydrate (mg/g FW)
Control	20.35±4.20a	21.72±3.97	2.99±1.59	45.46±9.77
Dummy	14.66±1.86ab	16.52±1.73	2.36±0.23	33.85±3.82
Net	14.39±0.54b	16.86±0.17	3.78±0.99	35.37±0.55
Significance[a]	*	ns	ns	ns

[a]*ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 8. Content of Vitamin C, total chlorophyll, carotenoid and nitrate in leaves of rocket grown in fully controlled environment under PVPs

Treatment	Tot Pool AscA (mg/g FW)	Total Chlorophyll content (mg/g FW)	Total Carotenoid content (mg/g FW)	Nitrate (ppm)
Control	2.30±0.54	0.62±0.10	0.19±0.03	113 ±29
Dummy	1.62±0.15	0.64±0.06	0.19±0.01	699±362
Net	1.94±0.35	0.82±0.10	0.23±0.04	669±405
Significance[a]	ns	ns	ns	ns

[a]*ns, Nonsignificant*

Table 9. Content of Vitamin C, total chlorophyll, carotenoid and nitrate in leaves of spinach grown in fully controlled environment under PVPs

Treatment	Tot Pool AscA (mg/g FW)	Total Chlorophyll content (mg/g FW)	Total Carotenoid content (mg/g FW)	Nitrate (ppm)
Control	1.24±0.31	1.17±0.05	0.29±0.01	488 ±73
Dummy	0.65±0.13	1.10±0.04	0.30±0.02	751±46
Net	0.51±0.23	1.09±0.16	0.27±0.05	547±107
Significance[a]	ns	ns	ns	ns

[a]*ns, Nonsignificant*

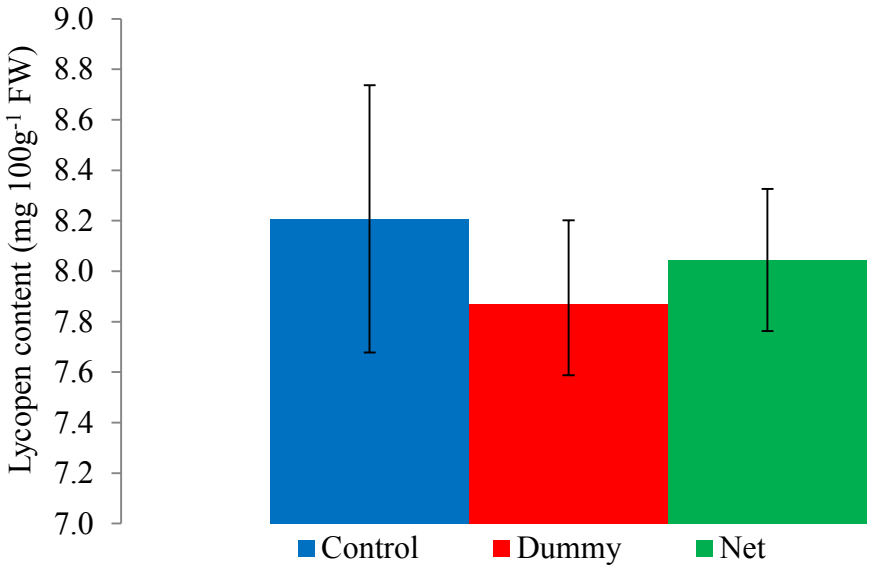

Figure 10. Lycopene content in fruits of tomato grown in fully controlled environment under PVPs

Table and figures of plants cultivated in greenhouse prototypes: Experiment 2

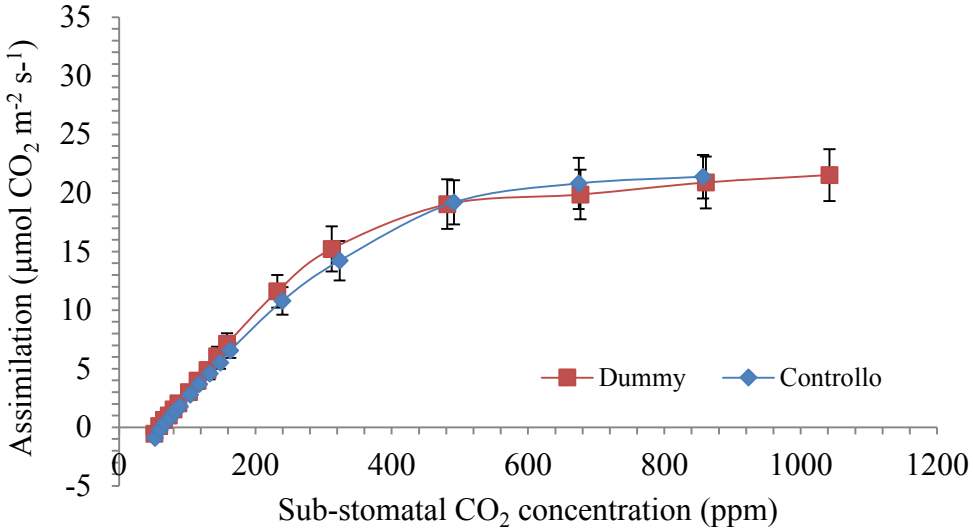

Figure 11. Assimilation vs Sub-stomatal CO2 concentration in tomato

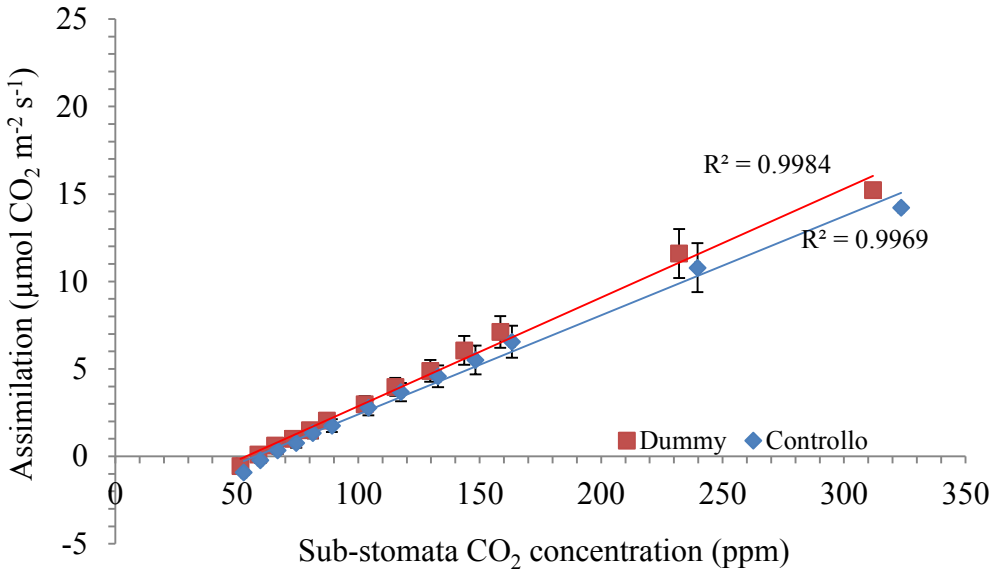

Figure 12. RuBisCo activity related to the slope of the curve in leaves of tomato grown in greenhouse prototypes

Table 10. Growth analysis and productivity of tomato plants grown in greenhouse prototypes

Treatment	Fruit yield (g plant^{-1})	Red fruits (n plant^{-1})	Fruit DM (%)	Shoot FW (g plant^{-1})	Root FW (g plant^{-1})
Control	8.55±2.21	3.33±0.80	14.25±0.20	26.14±3.10	3.63±0.62
Dummy	7.70±1.57	3.17±0.83	13.21±023	25.65±2.00	3.56±0.30
Significance[a]	ns	ns	**	ns	ns

[a]*ns, *,**, Nonsignificant or significant at P < 0.05 and P<0.01, respectively.*

Table 11. Carbohydrate content in fruits of tomato grown in greenhouse prototypes

Treatment	Glucose (mg/g FW)	Fructose (mg/g FW)	Sucrose (mg/g FW)	Starch (mg/g FW)	Total Carbohydrates (mg/g FW)
Control	20.02±0.83	24.69±0.90	3.31±0.28	1.61±0.18	49.63±1.90
Dummy	15.09±0.56	20.03±0.62	2.99±0.21	0.91±0.22	39.02±1.17
Significance[a]	***	***	ns	*	***

[a]*ns, *,***, Nonsignificant or significant at P < 0.05 and P<0.001, respectively.*

Table 12. Acidity and lycopene content in fruits of tomato grown in greenhouse prototypes

Treatment	Tot acidity (% Citric Acid)	Total Nitrogen in Red Fruits (mg/g DW)	Lycopene content (mg/100g FW)
Control	0.66±0.33	0.025±0.0004	9.39±0.31
Dummy	0.65±0.06	0.031±0.0025	9.13±0.34
Significance[a]	ns	ns	ns

[a]*ns, Nonsignificant*

Zeolite Potential Application in life Support

Introduction

The goal of this experiment is to test if type of zeolite have different effects on Rocket (*Eruca sativa* M. cv 'Rucola coltivata' provided by Semitalia snc, Terni, Italy). Zeolites exchange ions with the surrounding environment affecting the poll of nutrients available to plant in the substrate. Because of that, it is important to have deep understanding on the quality and the quantity of extra ions that must be added in order to provide the plants with the right supplement of nutrients.

Zeolites formed millions of year ago from the re-crystallization of volcanic ashes in the presence of water. They are hydrated aluminosilicate minerals belonging to a class of mineral named tectosilicates (*70*). The presence of pores, gives the zeolite high water retention capacity and its slight negative charged, due to the presence of Al ion in the lattice that helps to gradually release cations (like calcium and ammonium) in the rootzone. This characteristic curbs losses of nutrients for leaching. The negative charge of zeolite has positive effects on the quality of vegetable production even if cultivated on polluted soils. In fact, many of the polluting heavy metals and metals in general that promote pests and diseases are positive charged as Pb^{+2}, Cu^{+2}, Fe^{+3} and Cr^{+3} (*71*). They are weakly bound by the zeolitic lattice and released step by step.

Material and methods

Six growth modules provided by Aero Sekur S.p.a were clustered in 2 groups and accommodated into two twin growth chambers (Fitotron SGD170 Sanyo Gallenkamp UK) as shown in Figure 13 to ensure constant air humidity of 70 %, constant 400 ppm of CO_2, 25 °C during the day and 20°C at night. Blue (450 nm) and red (660 nm) light was supplied using 2 different LED bars provided by RL3 S.p.a. Roma of 10 Watt each for 12 hours a day mounted on each growth module. The main body of the growth module was made of Poly(methyl methacrylate) or PVC and it could accommodate 15 l of nutrient solution. A diving pump delivered the nutrient solution within the upper container at root level where zeolite was located. Excessive nutrient solution flowed back to the reservoir via the hole deliberately made at the bottom of the container. Carriage of debris from the upper container to the main body was prevented by filters. The irrigation system was programmed to deliver the nutrient solution for an hour every 3 hours. It was activated soon after the sowing. No transparent materials were used in order to keep the nutrient

solution in complete dark throughout the experiment. Soil moisture, soil temperature, air temperature and light incidence (considered as Photosynthetic Active Radiation) were measured by sensors located both within the zeolite and outside the growth module. Data were recorded every 15 minutes from sowing to harvesting for all growth modules by a data logger WatchDog 1000 series and downloaded weekly.

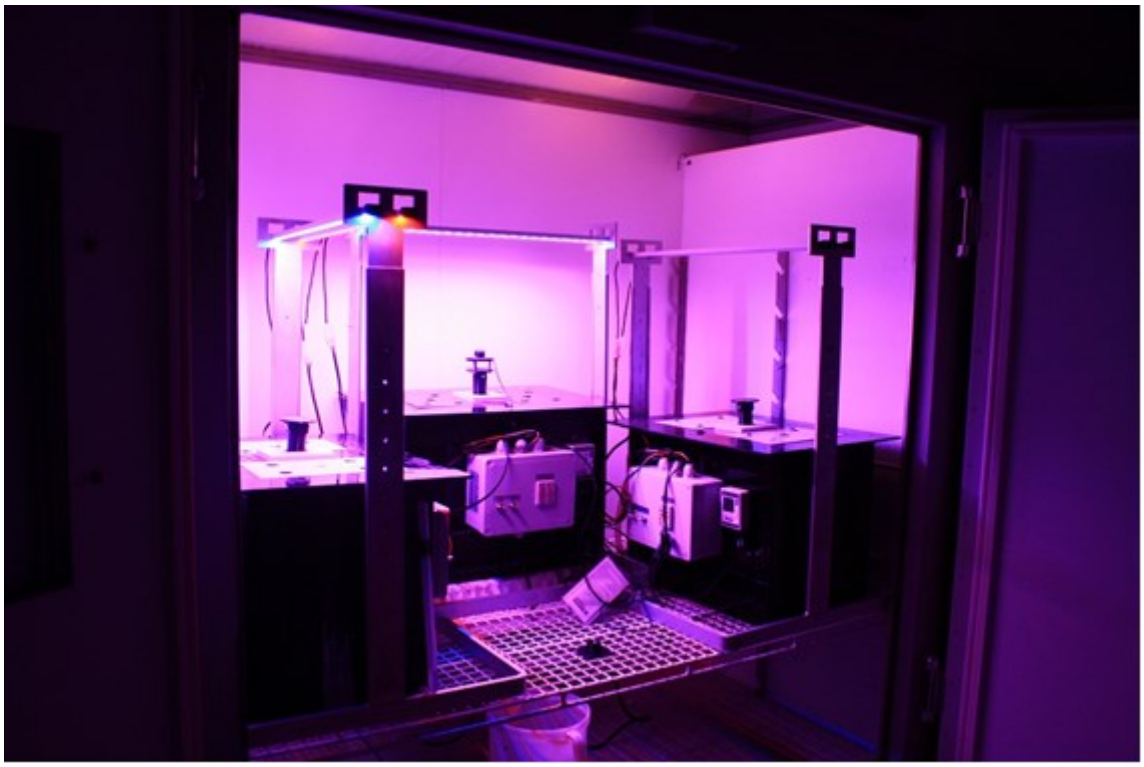

Figure 13. Accommodation of the growth modules into the cabinet

In this experiment, Chabazite and Clinoptilolite are the two types of zeolites tested in a system named Zeoponic. Zeoponic comes from the union of the words zeolite and hydroponic where the zeolite is the substrate that interacts with the nutrient solution affecting the availability of some ions for plants. Clinoptilolite was provided by IDROPONICA.IT via Bolognola, 30, Roma. Chabazite was produced by Aero Sekur S.p.a. using red tuff with black spots collected at "*Cava di tufo monte del tempio"* locality of Monte del Tempio, 01036 Nepi (VT), Italy. The rock was crushed mechanically into small pieces and sieved for homogenous 3 mm wide granules. Additionally, 40 % (v/v) of perlite was mixed with the two zeolites to completely fill up the tray. Each growth module accommodated 15 liters of nutrient solution which contained (mg l^{-1}): $N\text{-}NO_3$ (196), $N\text{-}NH_4$ (14), $S\text{-}SO_4$ (30), P- H_2PO_4 (31), K (234), Ca (160), and Mg (48), Fe (0.6), Mn (0.5), B (0.5), Cu (0.02), Zn (0.05), and Mo (0.01). The initial EC and pH of the nutrient solution were 1.8 dS m^{-1} and 6.0, respectively (*72*). At the end of the experiment,

residual volume of nutrient solution was recorded as well as EC and pH. Both parameters were measured using a portable pH/EC/TDS/Temperature meter model HI 98129 by Hanna Instrument.

Germinability. Seeds were sterilized with NaClO 2 % for 10 min then rinsed 3 times with deionized water. Consequently, sixty seeds per module were divided into 20 clusters of 3 seeds each and gently laid on a small loose pad of sterile cotton previously dampened with deionized water. Periodically, deionized water was pipetted on pads in order to keep the seeds moist. The number of seeds completely germinated was recorded on the seventh day from sowing. Seedlings must have been dark green colored with both hypocotyl and root well developed in order to be considered valid for germinability. Germinability was calculates as: percentage of seeds well germinated/total seeds sowed. After the count, only 20 plants per growth module were left growing in order to avoid intraspecific competition.

Fluorescence and Photosynthesis. Measurements were taken on homogenous and fully expanded leaves at six weeks from sowing. Six values per treatments were always recorded. Assimilation rate (A) of CO_2 and stomatal conductance (gs) were measured using a portable gas exchange system LI-6400xt LI-COR equipped with leaf chamber fluorometer cuvette (mod. LCF-1005). Two different light intensities were used for the gas exchange measurement: a) ordinary light (OL) of 65 μE m^{-2} s^{-1} that was the mean intensity of incident light received by plants throughout the growth; b) saturating light (SL) of 800 μE m^{-2} s^{-1} previously determined averaging the minimum intensity of light required for saturating the photosystems in 4 different plants randomly chosen. Once clamped, the leaf was first left acclimate to OL, and then values were recorded. Afterwards, the light intensity was switched to SL and the data were recorded as soon as the leaf acclimated. Six values per treatment were taken. Fluorescence was measured using a portable Walz Mini-Pan-II. Fluorescence in dark adapted leaves was measured after 30 min of adaptation to complete dark (*73*). The efficiency of Photosystem II (PSII) was calculated as F_v/F_m where (F_v) was the difference between the fluorescence emitted by PSII reaction centers when maximally open (F_m) and the fluorescence emitted by the same PSII reaction center when maximally closed (F_0). The florescence on light adapted leaves was measured after few hours of exposure. Again, the efficiency of PSII when leaves are adapted to light was calculated as F_v'/F_m' where F_v' was the difference between

the fluorescence emitted by PSII reaction centres when maximally open (F_m') and the fluorescence emitted by the same PSII reaction centre when maximally closed (F_0') (*74*).

Plants were harvested 65 days after sowing for growth analysis and quality analysis. Six plants per treatment were harvested, weighted and put into a ventilated oven at 80°C till constant weight. The dry weight was recorded. The number of leaves per plant was recorded as well as the total leaf surface using a Delta T Devices-UK Area meter. Leaves from other six plants per treatment were finely grounded in liquid nitrogen with chilled mortar and pestle for analysis of: non-structural carbohydrates (glucose, fructose, sucrose and starch), chlorophyll and carotenoid content, soluble and non-soluble proteins, nitrate and malic acid measured as described in General methods.

Results and discussion

The percentage of seeds germinated in clinoptilolite and chabazite was very: 53 ± 3.3 and 60 ± 6.7 respectively. The differences between the substrates in terms of chemistry and structure were not sufficiently effective in altering the germinability of seeds of rocket. However, in both substrate the germinability was lower than found in laboratory experiments assessing the germinability of rocket seeds (data shown in the next experiment, (Figure 17). The assimilation rate was low in both treatments but it is unlikely due to substrate effects due to the low light intensity provided by LED bars. Although the efficiency of photosystem II was high, very close to maximum theoretical value, plants did not perform efficiently in the organication of carbon. The photochemical processes involved in the harvesting of light and the conversion of its electromagnetic energy into biochemical energy as highlighted by Chlorophyll *a* fluorescence analysis (Table 13) was efficient, but the quantity of atmospheric carbon reduced to organic carbon was low (Table 13). The growth was visibly slow and plants needed far longer time than usual to reach a size commercially valid. Moreover, it was found 800 $\mu E\ m^{-2}\ s^{-1}$ to be sufficient to saturate the photosynthetic apparatus. At saturation point the plant cannot increase the quantity of carbon reduced at the increasing of light availability (*64*).

Mineral analysis of clinoptilolite and chabazite were done before and after the experiment for a complete overview of the ion exchange among the system zeolite, nutrient solution and plants. The release of minerals to plants was quite similar between the two zeolites, chabazite lost more manganese that clinoptilolite during the growth cycle of rocket. Chabazite generally reduced biomass production but only dry mater of roots was

statistically different and shoot/root ratio consequently. Overall quality of the leaves was influenced by different substrates. Nitrate content was high in both treatments while ascorbic acid content was rather low. Carbohydrate and protein content was also low in both treatments.

Conclusions

Growth modules permitted the growth of rocket plants from seeds to harvest automatically. In addition, 15 litres of nutrient solution were sufficient to support the complete growth of 20 plants per module without signs of wilting or salinity stress. Both clinoptilolite and chabazite exchanged ions with nutrient solution and plants, as demonstrated in

Table 14 by the decreasing in the concentration of some metals like aluminium, zinc and manganese at the end of the experiment. Despite very little differences, there was no evidence of effects on growth and food quality due to the use of different zeolites apart from chlorophyll *b* and carotenoid content as shown in

Table 16. Higher Vitamin C and lower nitrate content are always desired: usually ascorbic acid in rocket ranges from 0.8 mg g^{-1} to 1 mg g^{-1} (*75*) that is higher than found in this experiment (Table 17) while nitrate content should be as low as possible being them a precursor of toxic compounds once ingested (*76*). The latter is considered as anti-nutritional factor. According to EFSA (2011) (*77*) nitrate content in leaves of rocket should not exceed 6000 ppm for cultivation from 1 April to 30 September and 7000 ppm for those occurring from 1 October to 31 March. Nitrate content of rocket cultivated in growth module was higher than the threshold, Table 17. High content of nitrate, low content of carbohydrates and ascorbic acid along with longer period of growth are factors that can be associated to low light availability. In fact, for this experiment the light was rather low, approximately 65 μE m^{-2} s^{-1} with saturation point at 800 μE m^{-2} s^{-1}. Primary and secondary metabolites strictly depend on energy source as quantity and quality. Two wavelengths were used in the blue and red spectra of 10watt each. Although they are the most influential portion of the visible light spectrum on photosynthesis, the total amount of μmol of photons that reached plants was very low, slightly above the compensation points. The compensation point is the threshold where the quantity of carbon emitted with

respiration equals the quantity of carbon that fixated via photosynthesis and available for biomass production. Therefore, if most of the carbon reduced during the photosynthesis is quickly oxidized during the respiration, the investment in metabolites is low. Hence, the general low quality of rocket.

In conclusion, clinoptilolite and chabazite did not affect germinability, yield and quality of rocket cultivated in growth modules supporting the hypothesis to use zeolites for life support systems.

Tables

Table 13. Assimilation rate and fluorescence on leaves of rocket grown on different zeolites

Substrate	Assimilation rate (μmol CO_2 m^{-2} s^{-1})		Fluorescence	
	Growth light 65 μE m^{-2} s^{-1}	Saturating light 800 μE m^{-2} s^{-1}	Dark adapted leaves Fv/Fm	Light adapted leaves Fv'/Fm'
Clinoptilolite	3.1 ± 0.15	13.6 ± 1.45	0.84 ± 0.003	0.76 ± 0.007
Chabazite	2.7 ± 0.18	13.7 ± 1.34	0.81 ± 0.008	0.77 ± 0.010
Significance[a]	ns	ns	*	ns

[a]*ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 14. Mineral composition of zeolites at the beginning and at the end of the growing cycle of rocket

Parameter	Before planting		After harvesting	
	Clinoptilolite	Chabazite	Clinoptilolite	Chabazite
Total nitrogen (% DW)	<0.3	<0.3	<0.3 ± 0.01	<0.3 ± .01
Phosphorus (mg/kg DW)	37.9	45.2	38.5 ± 2.75	36.6 ± 0.1
Potassium (% DW)	1.6	1.8	1.81 ± 0.025	1.85 ± 0.05
Chlorine (% DW)	0.56	0.45	0.52 ± 0.037	0.56 ± 0.115
Sodium (% DW)	0.36	0.29	0.34 ± 0.022	0.36 ± 0.075
Iron (% DW)	1.9	2.0	1.65 ± 0.05	1.87 ± 0.1
Manganese (mg/kg DW)	485.8	785.7	474 ± 80	668 ± 101
Zinc (% DW)	71.6	68.3	55.5 ± 5.12	65.1 ± 1.45
Copper (mg/kg DW)	4.2	8.3	4.05 ± 2.32	8.75 ± 0.75
Aluminum (% DW)	3.0	4.5	2.81 ± 0.98	4.03 ± 1.63

Table 15. Growth analysis of rocket grown on different zeolites

Treatment	Root DW (g plant^{-1})	Leaf FW (g plant^{-1})	Leaf DW (g plant^{-1})	Leaf (cm^2 plant^{-1})	SLDW (mg cm^{-2})
Clinoptilolite	0.10±0.04	16.67±1.43	0.97±0.10	353±38.55	14.46±0.81
Chabazite	0.08±0.02	15.46±1.97	0.84±0.14	320±37.90	15.78±0.61
Significance[a]	ns	ns	ns	ns	ns

[a]*ns, Nonsignificant*

Table 16. Chlorophyll a, Chlorophyll b and Carotenoids content in leaves of rocket grown on different zeolites

Treatment	Chlorophyll*a* (mg/g FW)	Chlorophyll*b* (mg/g FW)	Total Chlorophyll content (mg/g FW)	Total Carotenoid content (mg/g FW)	Ch*a*/Ch*b*
Clinoptilolite	0.31±0.02	0.13±0.01	0.44±0.03	0.19±0.017	2.39±0.14
Chabazite	0.27±0.01	0.10±0.01	0.37±0.02	0.15±0.003	2.77±0.09
Significance[a]	ns	*	ns	*	ns

*[a]ns, *, Nonsignificant or significant at P < 0.05, respectively.*

Table 17. Content of organic acids, nitrate and proteins in leaves of rocket grown on different zeolites

Treatment	Tot Pool AscA (mg/g FW)	Nitrate (ppm)	Malic acid (mg/g FW)	Total protein content (mg/g FW)	Soluble protein content (mg/g FW)
Clinoptilolite	0.62±0.05	7719±223	1±0.08	5.89±0.45	4.31±0.35
Chabazite	0.63±0.02	7889±197	0.83±0.05	5.42±0.19	4.20±0.20
Significance[a]	ns	ns	ns	ns	ns

[a]ns,Nonsignificant

Table 18. Content of carbohydrates measured in leaves of rocket grown on different zeolites

Treatment	Glucose (mg/g FW)	Fructose (mg/g FW)	Sucrose (mg/g FW)	Starch (mg/g FW)	Total Carbohydrate (mg/g FW)
Clinoptilolite	0.90±0.09	0.09±0.02	0.64±0.07	0.16±0.02	1.79±0.14
Chabazite	1.14±0.10	0.10±0.02	0.68±0.06	0.22±0.02	2.14±0.18
Significance[a]	ns	ns	ns	ns	ns

[a]ns,Nonsignificant

Effects of methanol, ethanol and isopropanol on germinability, physiology and quality of rocket in the short-term exposure

Introduction

Long term manned missions in next decades are planned to reach, by a stepwise approach, Moon and Mars and eventually to set manned premises in these far and inhospitable environments. This kind of mission will require an high level of Biorigenerative Life Support Systems (BLSS) for gas, water and food recycling (*78*). In (BLSS) higher plants play fundamental roles in many aspects: food production, water recycling, oxygen production, reduction of carbon dioxide, maintenance of crew's wellbeing, thermal and air humidity control. Cropping in fully controlled environments is always challenging and once in space, it could be even more difficult. Proper understanding of the effects of environmental factors on plants could be vital for plant productivity and hence, for the astronauts survival. Losses of food or production of non-edible biomass must be avoided. *Ad hoc* manipulation of environmental factors can prevent these problems and in some cases, it can accelerate plant growth, productivity and quality.

In a closed extra-terrestrial BLSS where nothing can be wasted, biomass and water are precious resources that must be reused/recycled in order to reduce replenishments from earth that could be, in some cases, prohibitive. The possibility to reuse water in BLSS implies awareness of the quality of the water that is intended to be used, for example for cropping purposes. Some pollutants can be released during common daily astronauts' activities and gather into the water stream. Plants fed with polluted water can be damaged by pollutants which can be uptaken and accumulated along with toxic compounds in edible tissues putting under threat the crew's heath if astronauts fed on that food. Therefore, even the slightest accumulation of toxic compounds in plants should be avoided in order to keep the food as safe as possible.

Accumulation of pollutants in the Crew Latent Condensate (CLC) of the ISS is expected, as it is in other water recovery streams (*79*). Possible uses and composition of future water steams in still undefined BLSS is impossible at moment but the presence of a large number and variety of pollutants in such water imposes to consider what would be the effect of such pollutant on plant physiology, on food quality and safety and on the fate of

the pollutants when interacting with the plant metabolism. Although the bioactivity of some volatile alcohols was already tested on radish (*80*, *81*), literature still lacks of information regarding possible effects on horticultural species when organic pollutants, specifically MEI, are provided via nutrient solution.

The general aim of the experiments reported here was to study the short term effects of some of the pollutants present in the CLC of the ISS (namely methanol, ethanol and isopropanol MEI) when present in the nutrient solution of hydroponically grown rocket. Specific tasks were to evaluate i) the effects of MEI on rocket seed germination ii) effects of MEI on rocket physiological status iii) the intake, translocation, accumulation, metabolism and release in the atmosphere of MEI and their metabolic products.

Material and methods

Germinability. Hundred seeds of rocket *(Eruca sativa* M. var "rucola coltivata") for each of four replicates per treatments were positioned in 8 cm Ø Petri dish sterilized under UV light (Mini Flow sterile laminar flow hoods - PBI Spa Italy) loaded with two layers of filter paper. Germination was initiated by adding 1 ml of deionized water for controls and a 1% alcoholic solutions for experimental treatments. Four treatments were applied: a) 1% methanol. b) 1% ethanol c) 1% isopropanol d) 1% of a solution of the three alcohols in a 6-14-80 % ratio as reported for CLC stream (*79*). The effect of ethanol was also investigated at 0.5%, 0.2% and 0.8 $x10^{-4}$ %. Alcohols were provided by Sigma Aldrich. Germination was allowed at room temperature (Figure 15) and seedlings were counted after 7 days, according to official methods released by the Italian *Ministero delle Politiche Agricole, Alimentari e Forestali.*

Growth conditions and physiological status measurements. Plants for the assessment of the physiological response to short-term exposure, were grown from seeds in Eppendorf tubes. The tubes, whose bottom was cut to allow roots exit, were filled with sterile vermiculite Figure 16. Eppendorfs were mounted in a plastic frame and put into 1.5 l plastic box in a flooding system. Eppendorf tubes and boxes were carefully black painted in order to prevent algae formation during the experiment. Oxygenation was provided in each box by bubbling air with dedicated air pumps. Boxes were filled with distilled water during the germination that was completed in a week. After germination, water was replaced with standard Hoagland solution 1.5 dS m^{-1} pH 6.0. Nutrient solution was changed once a week and daily refilled with distilled water. Boxes were positioned in a

growth cabinet (Fitotron SGD170 Sanyo Gallenkamp UK) at constant 25 °C, 600 PAR, 12 h day/night cycle, 400 ppm CO_2. Plants were used for experiments 5 weeks after sowing. Gas exchanges and chlorophyll *a* fluorescence measurements were performed 24 h after MEI feeding (1%). See general methods for description of procedures.

Measurement of alcohol accumulation in leaves. Accumulation of methanol, ethanol and isopropanol, after feeding experiments on detached leaves was measured, by Ionic Chromatograpy (Dionex TM ICS 5000). Fully expanded leaves of 5 weeks old plants were cut under water after two hours from the onset of the photoperiod and fed water for control or 5% alcohols solution. Leaves were left in feeding solution for 6 h in the same condition used for growth. Evaporation from the feeding vials was avoided by stretching a strip of Parafilm around the leaf petiole to seal the vials. Leaf discs were sampled with a cork borer and approximately, 100 mg of fresh tissue were extracted with a glass homogenizer in 1 ml of distilled water, the extract was centrifuged at 14000 g for 5 minutes for clarification and un aliquote of the supernatant was filtered prior of injection. (0.2 µm nylon syringe filters Whatman GE Healthcare Life Sciences USA).

Emissions of MEI and derived volatile metabolites. Two hours after the beginning of the photoperiod, the nutrient solution in 5 weeks old plants was replaced with water. MEI were purchased from Sigma Aldrich (USA). A leaf among the most expanded ones was selected and enclosed into a glass cuvette without detaching it from the plant avoiding damage to the petiole or the lamina, that would cause organic volatile compounds (VOC) emission. The leaf area enclosed in the cuvette, was measured at the end of the experiment. Pollutant free air was generated by mixing measured fractions (80% N_2, 20% O_2 and 400ppm CO_2) of ultra-pure gasses from cylinders, by mass flow controllers (Brooks Instrument). The circuit was maintained in a small overpressure condition in order to avoid VOC contamination from the ambient.

Light, at the intensity of 600 µ mol m^{-2} s^{-1} at the leaf level, was provided by 400 W HQI Osram (Germany) lamps. Cuvette outflow was connected to a PTR-MS (Proton Transfer Reaction Mass Spectrometer, Ionicon, Innsbruck, Austria) with a short Teflon (PTFE) tube with 1/4" of inner diameter. Sampling flow was 0.14 l min^{-1}. The ionization energy E/N (E is the electric field and N is the number of density of the drift tube molecules) was set at approximately 120 Td. Drift tube pressure, temperature and voltage were 2.20 mbar,

40°C and 600 V respectively. Mass spectrometric data were collected over a mass range of m/z 18-137 with a dwell time of 100 ms.

The relative humidity of air entering the cuvette was maintained constant at 50% by conditioning the temperature of the air stream (and hence its water content) with a thermos-regulated water bath. Light, at the intensity of 600 μ mol m^{-2} s^{-1} at the leaf level, was provided by 400 W HQI Osram (Germany) lamps. Cuvette outflow was connected to a PTR-MS (Proton Transfer Reaction Mass Spectrometer, Ionicon, Innsbruck, Austria) with a short Teflon (PTFE) tube with 1/4" of inner diameter. Sampling flow was 0.14 l min-1. The ionization energy E/N (E is the electric field and N is the number of density of the drift tube molecules) was set at approximately 120 Td. Drift tube pressure, temperature and voltage were 2.20 mbar, 40°C and 600 V respectively. Mass spectrometric data were collected over a mass range of m/z 18-137 with a dwell time of 100 ms. Ethanol, acetaldehyde and acetic acid were detected as m/z 47, 45 (*82*) and 61 (89%) (*83*) respectively. Methanol, formaldehyde and formic acid were detected as m/z 33 (*84*), 31 (*85*) and 47 (*82*) respectively. Isopropanol was detected as m/z 43 (73%) (*86*). Before starting with data records, daily calibration of the instrument was always performed using calibration gases provided by SIAD (Bergamo, Italy). Further details regarding theoretical and practical aspects of PTR-MS technique are reported by Lindinger et al. 1998. Simultaneously, gas exchange measurements where taken on a companion leaf of the same plant using a LI-6400xt LI-COR equipped with leaf chamber fluorometer cuvette (mod. LCF-1005) data were recorded automatically every 2 minutes for 3 hours. Cuvette conditions of humidity and temperature were maintained constant at 50% and 25°C respectively. After 20 minutes adaptation, alcohol (MEI were purchased from Sigma Aldrich USA) was injected in water at final 15 mM concentration. VOCs emissions where also detected 24 hours after the feeding applying the same set up on the same leaves for 30 minutes. Between the first and second set of measurements, plants were maintained at growth conditions and alcoholic solution in the box was neither replaced nor replenished. Emission rates after the feeding are reported as per unit of mol of water transpired.

Stable carbon isotope (^{13}C) labeling of alcohols and $^{13}C/^{12}C$ measurement in the respiratory CO_2. ^{13}C labelled alcohols were used to track the respiratory emission of carbon derived from fed alcohols. ^{13}C MEI (99%) were purchased from Sigma Aldrich (USA) and used in a 1:10 ratio with non-labelled MEI in the feeding solutions. After two

h into the photoperiod, 5 weeks old plants, were used for feeding experiments, by replacing the normal nutrient solution with distilled water, and alcohols solutions at 15 mM concentration. This procedure was applied separately on three replicates for each alcohol tested. Air bubbling was suspended during the feeding procedure to avoid fast evaporation of the alcohols. Fed plants were left in the growing chamber, solution was refilled as necessary, and after 24 h leaf discs (1.34 cm^2) were harvested from fully expanded fed leaves, inserted in 12 ml vials covered with aluminum foil to avoid photosynthetic CO_2 fixation. The CO_2 within the vial was sampled once every 30 min during a 3 hours period, results of the 6 measurements on the same vial were almost identical and third average was calculated ad used as the value of that measurement. At the end, leaf samples were freeze dried for dry weigh. Analyses of $^{13}C/^{12}C$ isotope ratios on CO_2 sampled from the vials were performed using an isotope ratio mass spectrometer (Isoprime, GV, Cheadle, UK) on line with the MultiPrep Bio system. This preparatory system allowed timely repeated collections of discrete amounts of CO_2 emitted by a leaf portion enclosed in the vial. Carbon isotope composition was calculated according with (*88*) as:

$$\delta C = (Rs/Rstd) - 1$$

where Rs and Rstd are the isotope ratio ($^{13}C/^{12}C$) of samples and of the international standard Vienna-Pee Dee Belemnite (VPDB). The standard deviation of replicate measurements was 0.1‰ for ^{13}C.

Results and discussion

Effects of MEI on rocket seed germination. Ethanol was the only alcohol that inhibited the germinability of seeds of rocket Figure 17, at 1% concentration. Further investigations have contributed to assess what is the minimum concentration of ethanol sufficient for inhibiting the germinability of rocket seeds. According to Figure 18 0.5% is still enough for inhibition, 0.2% reduced the germinability to 85% whilst $8 \cdot 10^{-4}$% (same concentration found in CLC) had not inhibitory effects. These results show that the concentration of MEI found in the CLC are not high enough to cause problems to the rocket seed germination. Seeds might experience various degree of O_2 availability during germination and are generally able to activate anaerobic metabolism, ethanol production and catabolism via alcohol dehydrogenase (ADH, EC 1.1.1.1), that is normally present and

can be activated in seeds. ADH catalyses the first step on alcohol metabolism in plants and can oxidase methanol, ethanol and isopropanol although at different rates (*89*). It is then not surprising that low concentration of alcohols were not toxic for rocket seeds.

Effects of MEI on rocket physiological status. 24 h after feeding, gas exchange showed only minor changes in MEI fed plants (data not shown). Chlorophyll a fluorescence analysis indicated that the overall efficiency of photosystem II (PSII) was only marginally affected by MEI Figure 19. Small changes recorded for the ethanol fed plants were not sufficient to reduce photosynthesis at growing light intensity. Assimilation rate was similar among treatments, pretty stable and close to 20 µmol CO_2 $m^{-2}s^{-1}$ for at least 90 minutes of measurements (data not shown).

Intake, translocation, accumulation, metabolism and release in the atmosphere of MEI and their metabolic products. Alcohol analysis in leaf tissues after feeding showed an accumulation of 0.9 % and 0.1 % of the fresh weight for of methanol and ethanol respectively, in rocked leaves (Figure 20). No isopropanol was detected. Leaves were fed 6 h with 5% MEI solutions, under growing conditions. Rocked leaves under the used growing conditions have an average fresh weight of about 380 g m^{-2}, with a water content in between 80-85%. Transpiration can be assumed to be on average around 3 - 4 mmol m^{-2} s^{-1}. This indicates that during the 6 h of 5% alcohols solution feeding, the rocket leaves should have transpired an amount of water equivalent to several times their water content. If the rocket leaves were "neutral" with respect to the intake, metabolism and release in the atmosphere of the alcohol, we should have found a much higher alcohol concentration in leaves. Hence from these data we can conclude that rocket takes up ethanol and methanol (see further data for isopropanol absorption) from the nutrient solution, translocate them to the leaves but dos not accumulate any of the fed alcohols in the edible plant parts.

The set up used in the present experiment allowed the on-line measurement of short chain oxygenated VOC (oxVOC) emission by intact rocket leaves during a feeding experiment with the full control of the environmental conditions surrounding the leaf. Meanwhile a side measurement allowed to measure the water transpiration rate of a twin leaf of the same plant, maintained at the same external environmental conditions as the one used for measuring oxVOC release. Hence it was possible to estimate the water and oxVOC emission rate under fully comparable conditions and then to estimate the molar ratio of

oxVOC emission versus water vapour emission of the fed plant. This is relevant to normalize the oxVOC emission to the stomatal conductance. Stomata are the most relevant way of oxVOC emission by leaves (*90*). On-line experiments under full environmental control, are crucial to track the fate of MEI in the plants because oxVOCs production and metabolism is strongly dependent on plant response to the environment (*90*).

MEI feeding affected plant oxVOC emission in a matter of minutes (Figure 23, Figure 25 and Figure 27). Emission of oxVOC prior of MEI feeding was very low, so that the effect of MEI feeding on oxVOCs emission was clearly recorded by the PTR-MS system. Methanol feeding resulted in an almost linear increase of the methanol emission for at least 150 minutes. The emission of formaldehyde (FA) and acetic acid were also checked but no relevant changes in emission were found (Figure 23). After 24 h of feeding leaves still emitted 8 times more methanol than the control leaves (Figure 24) and still no relevant amount of FA and acetic acid (data not shown). Ethanol feeding resulted in a fast emission of acetaldehyde (AA) and minor, but still measurable amount of ethanol, while non changes in acetate emission were recorded. Emissions of AA increased in time for at least 150 min. After 24 h of feeding AA emission was still extremely high with respect to the emission of control leaves, although lower than the one recorded at the beginning of feeding. Ethanol and acetate emissions where similar to control. Isopropanol feeding caused a rapid and linear increase of propanol emission in the gas stream. The emission was still high 24 h after feeding. These results prove that isopropanol was taken up by leaves as the other MEI tested, then since no isopropanol was detected in leaf tissues by Ion Chromatography, we can conclude that even isopropanol was taken up by the root system as ethanol and methanol and that even isopropanol does not accumulate in edible plant biomass.

Feeding rocket plants with ^{13}C labelled MEI caused measurable increases of the δC, of the CO_2 emitted by leaves, consistent with MEI carbon being oxidised to CO_2. Changes in δC where recorded as soon as 30 minutes after the start of feeding for methanol and ethanol. After 24 h of feedings changes of δC were evident for all MEI although with different intensities (methanol > ethanol > isopropanol). Even in the case of isopropanol, where the δC change appears small it should be consider sufficient to show an increased presence of ^{13}C in the emitted CO_2 after feeding. These results, coupled with the results of oxVOC emission, are very relevant because they show that rocket is able not only to

take-up translocate and release MEI, but also that it can completely metabolise MEI to CO_2.

Conclusions

The aim of this work was not to produce a quantitative model of the fate of MEI when in contact with the rocket root system, but some quantitative considerations are possible. MEI/Water molar fraction in the feeding solution was 2.70 x 10^{-4}, while the estimated MEI/Water (or methanol + acetaldehyde in the case of methanol feeding) in the transpiration stream, was approximately half of that for all three MEI tested (Figure 23Figure 25 and Figure 27) in the period of measurements, and it was much lower after 24 h. Even if we lack information on possible limitation to MEI root absorption, our results together with that on modification of δC by ^{13}C MEI feeding, points to the ability of rocket to eliminate a relevant fraction of each MEI from the ambient. However the emission of acetaldehyde after feeding of ethanol is a negative aspect that should be taken into account for the role that this toxic compound in a closed atmosphere. However, MEI and their metabolites are highly soluble in water, then their evaporation from a water solution is lower than that of other less water soluble VOCs, and the equilibrium between emission and absorption can depend on physicochemical factors and not only on physiological ones. For this reason, high flux leaving the plant is expected to pass through stomata in response to a concentration gradient. Using pure gas for fluxing the experimental chamber as it was done in this experiment can alter the gradient with respect to the one caused by normal ambient air, and then cause an increase of emission (*91*). This is particularly relevant in the case of plant growing in space where the need for full environmental control can suggest to provide VOC free air to plants. That would stimulate the emission of oxVOC. It is also conceivable that the rate of oxVOC that we recorded in control plants was stimulated by the use of oxVOC free air**.**

There is a rising number of reports on plant-environment interaction in terms of short chain oxVOC (methanol, ethanol, formaldehyde, formic acid, acetaldehyde, acetic acid and acetone) (*90*, *91*). It is recognised that the oxVOC production and destiny in the environment involves plants, soil and the atmosphere in a complex system of source and sink relationships, driven by metabolism of living organisms, the environmental conditions and the chemical functioning of the atmosphere. Hence this study on the fate of MEI in an hypothetic BLSS along with clues on the effect of MEI on food and environmental quality in space, provide new information on oxVOC-plant interaction.

This highlights the importance of oxVOC-plant interaction at the global level on Earth. In fact, there is a considerable lack of information about oxVOC and plants, particularly in relation to plant metabolism. Methanol and ethanol can be produced by plant metabolism and certainly, plants can be a source of these alcohols for release in the environment. On the contrary, there are no clear reports, on my knowledge, regarding the production of isopropanol by plants. On the other hands, all MEI can be oxidised by plant ADH via reversible reaction to yield formaldehyde, acetaldehyde and acetone although with rates generally higher for ethanol than for the other alcohols (*92*, *93*). Methanol feeding caused the release of methanol and not of formaldehyde, the product of ADH oxidation of methanol, nor formic acid, that can be generated by oxidation of formaldehyde. Formaldehyde (FA) is an high volume pollutant of indoor ambient, and plants are known for their ability to scavenge FA from indoor habitats (*94*). FA is normally present in plat metabolism, and it can be oxidized by a two steps process to CO_2 by FA-dehydrogenase (FDH E. C. 1.2.1.46) and formate dehydrogenase (FTDH E.C. 1.2.1.2) (*95*). Plant fed with gaseous ^{14}C-FA in the surrounding atmosphere, incorporated more than 75 % of FA carbon in metabolites and structural components in the light, suggesting that after entering the plant, FA was cleaved to CO_2 that re-entered the plant metabolism via photosynthetic CO_2 fixation (*95*). Our results are consistent with methanol being oxidized at a rate low enough to allow all produced FA to be oxidized to CO_2. It is very likely that this pathway was functioning in rocket as it is suggested by the change of δC after methanol feeding. It is noteworthy that the change in δC caused by methanol was the highest among MEI. Although after methanol feeding the most striking evidence was the emission of acetaldehyde, relevant change in δC was also recorded showing that an amount of acetaldehyde entered the respiratory metabolism. Limited flux of methanol to CO_2 was also found after ^{14}C methanol feeding to carrot tissue culture, that also accumulated acetaldehyde (*96*). Acetone is produced by plant metabolism (*91*) and plants are able to metabolize acetone to other plant metabolites, structural compounds and CO_2 (*97*). When fed with ^{14}C labeled acetone, different tissues partitioned approximately 20% of the fed radioactivity to CO_2 (*97*). When fed with isopropanol, rocket emitted isopropanol at high rates and δC changed only slightly. This is perfectly consistent with low ADH activity on isopropanol and with limited flux of the derived acetone to respiratory metabolism.

Finally, it should be noted that ADH activity in plants is subject to metabolic and environmental stimulation both at the biochemical and gene level (*98*) as are, very likely, other enzymes of the oxVOCs metabolism. Hence, the ability of plants to metabolise MEI, and the products of their oxidation, can vary when exposure is prolonged in time. Further studies on this aspect would be crucial for proper evaluation of MEI fate in real BLSS.

In conclusion, rocket seeds germination, plant growth and quality of rocket leaves (in terms of MEI accumulation) were not negatively affected by short term MEI feeding *via* the nutrient solution even at concentration far higher than that expected in the BLSS water recovery streams. MEI were absorbed via the root system, translocated to the shoot, partially metabolised and partially released *via* the transpiration stream. Evidences were produced about the ability of rocket to eliminate a relevant fraction of the MEI from the BLSS, without causing accumulation of MEI in the edible plant parts. However, the ability of rocket to rapidly metabolize ethanol to acetaldehyde and to release it in the transpiration stream, points to the role of plants as potential source of oxVOCs, an underestimated type of organic compound released by living organisms whose role on plant-environment interaction should be further studied both on the BLSS and on the Earth.

Tables and Figures

Component	CI	SMAC	MW	Percent Carbon [%C]	Loading Density [mg/L]	Percent of Stream [%]
2-propanol		×	60.1	60.0	46.297	18.6
1,2 propanediol			76.1	47.4	45.234	18.2
bicarbonate			61.0	19.7	33.170	13.3
acetic acid [ethanoic acid]		×	60.1	40.0	14.614	5.9
ammonium	×		18.0	0.0	13.527	5.4
caprolactam			113.2	63.7	11.834	4.8
ethylene glycol [1,2-ethandiol]		×	62.1	38.7	10.224	4.1
glycolic acid [hydroxy acetic acid]			76.1	31.6	10.194	4.1
ethanol		×	46.1	52.1	8.181	3.3
formaldehyde [methanal]		×	30.0	40.0	8.136	3.3
formic acid [methanoic acid]			46.0	26.1	7.239	2.9
propanoic acid			74.1	48.6	3.916	1.6
methanol		×	32.0	37.5	3.737	1.5
lactic acid [2-hydroxy-propanoic acid]			90.1	40.0	3.079	1.2
4-ethyl morpholine			115.2	62.6	2.516	1.0
urea			60.1	20.0	2.415	1.0
chloride	×		35.5	0.0	1.465	0.6
4-hydroxy-4-methyl-2-pentanone			116.2	62.0	1.247	0.5
2-butoxyethoxy-ethanol			162.2	59.2	1.130	0.5
4-acetyl morpholine			129.2	55.8	1.092	0.4
1-butanol		×	74.1	64.8	0.937	0.4
2-butoxyethanol			118.2	61.0	0.803	0.3
carbon disulfide	×	×	76.1	15.8	0.785	0.3
octanoic acid			144.2	66.6	0.665	0.3
zinc	×		65.4	0.0	0.650	0.3
N,N-dimethylformamide [N,N-dimethyl formic acid amide]			73.1	49.3	0.608	0.2
total protein			3,206.3	53.0	0.600	0.2
hexanoic acid			116.2	62.0	0.582	0.2
isocitric acid [1-hydroxy-1,2,3-propanetricarboxylic acid]			192.1	37.5	0.576	0.2
dibutyl amine			129.2	74.3	0.566	0.2
potassium	×		39.1	0.0	0.542	0.2
constituents totaling less than 5%					*9.546*	*3.8*
nitrite	×		*46.0*	*0.0*	*0.517*	*0.2*
2-ethoxyethanol		×	*90.1*	*53.3*	*0.504*	*0.2*
acetone [2-propanone]		×	*58.1*	*62.0*	*0.348*	*0.1*
magnesium	×		*24.3*	*0.0*	*0.282*	*0.1*
phenol		×	*94.1*	*76.6*	*0.204*	*0.1*
silver	×		*107.9*	*0.0*	*0.200*	*0.1*
acetaldehyde [ethanal]		×	*44.1*	*54.5*	*0.098*	*0.0*
cyclohexanone		×	*98.1*	*73.4*	*0.089*	*0.0*
nickel	×		*58.7*	*0.0*	*0.087*	*0.0*
acetophenone		×	*120.2*	*80.0*	*0.083*	*0.0*
calcium	×		*40.1*	*0.0*	*0.060*	*0.0*
sulfate	×		*96.1*	*0.0*	*0.052*	*0.0*
methylene chloride [dichloromethane]	×	×	*84.9*	*14.1*	*0.050*	*0.0*
manganese	×		*54.9*	*0.0*	*0.035*	*0.0*
methyl ethyl ketone [2-butanone]		×	*72.1*	*66.6*	*0.023*	*0.0*
iron	×		*55.9*	*0.0*	*0.008*	*0.0*
tetrachloroethene	×	×	*165.8*	*14.5*	*0.005*	*0.0*
copper	×		*63.6*	*0.0*	*0.004*	*0.0*
isobutyl methyl ketone [4-methyl-2-pentanone]		×	*100.2*	*72.0*	*0.002*	*0.0*
cadmium	×		*112.4*	*0.0*	*0.001*	*0.0*
lead	×		*207.2*	*0.0*	*0.001*	*0.0*
toluene		×	*92.1*	*91.2*	*0.001*	*0.0*
ethyl benzene		×	*106.2*	*90.5*	*trace*	*0.0*
benzene		×	*78.1*	*92.3*	*trace*	*0.0*
chloroform [trichloromethane]	×	×	*119.4*	*10.1*	*trace*	*0.0*
Total					**248.76**	**100**

Figure 14. Waste water contaminants in Crew Latent Condensate (*79*)

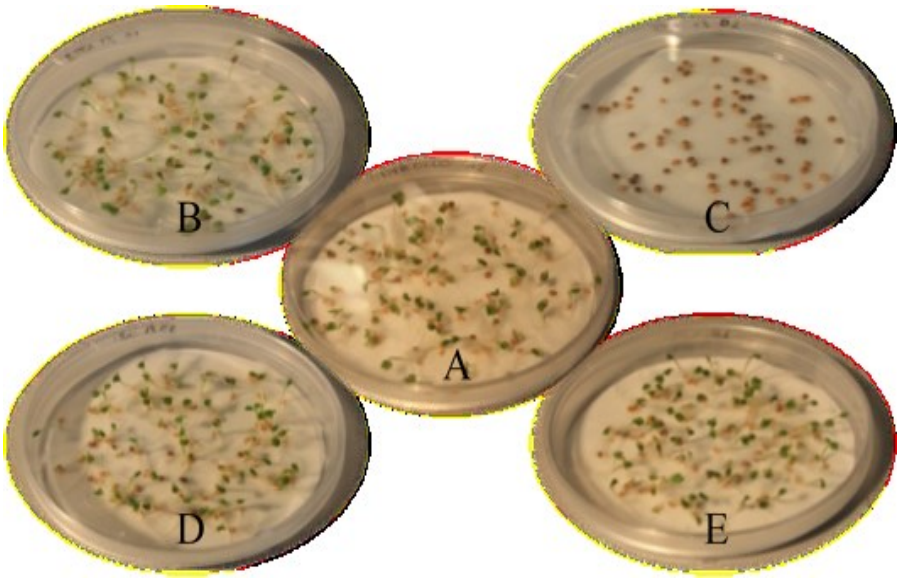

Figure 15. Germinability of seeds of rocket exposed to different 1% alcoholic solutions for 7 days: a) control; b) methanol; c) ethanol; d) isopropanol; e) mix

Figure 16. Plants of rocket grown from seeds into eppendorfs

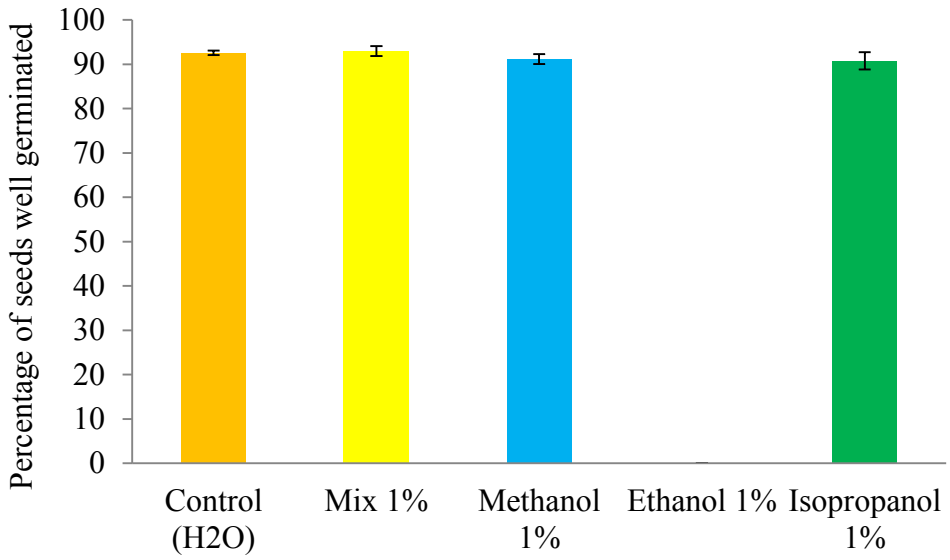

Figure 17. Effects of methanol, ethanol and isopropanol (1% v/v) on germinability of seeds of rocket after 7 days of exposure

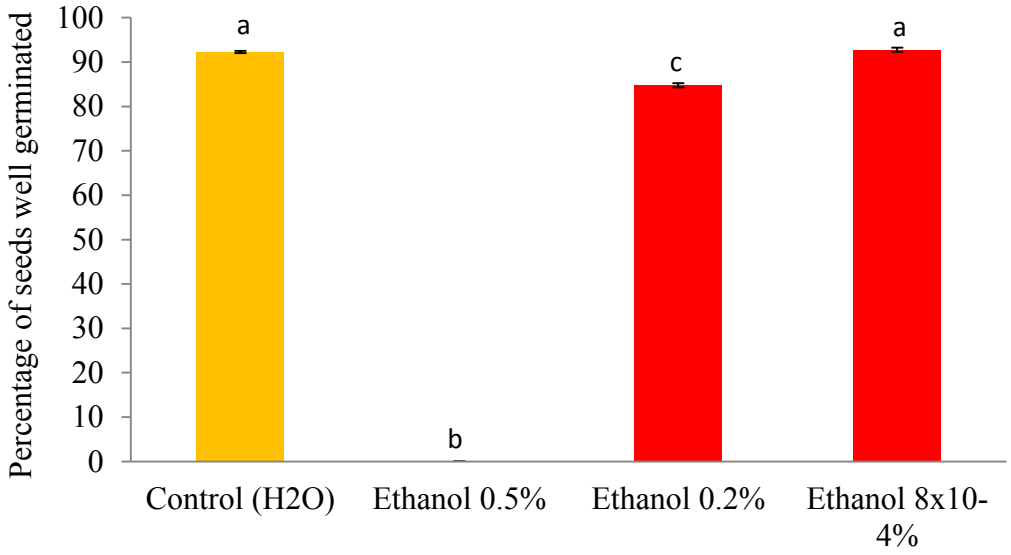

Figure 18. Effects of different concentrations of ethanol on germinability of seeds of rocket after 7 days of exposure

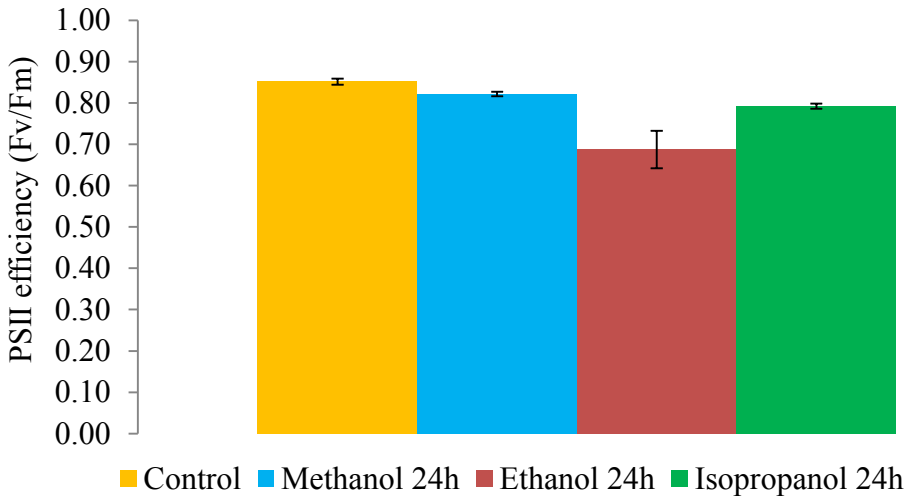

Figure 19. Photosystem II efficiency of leaves of rocket exposed to 1% (v/v) alcoholic solution for 24hours

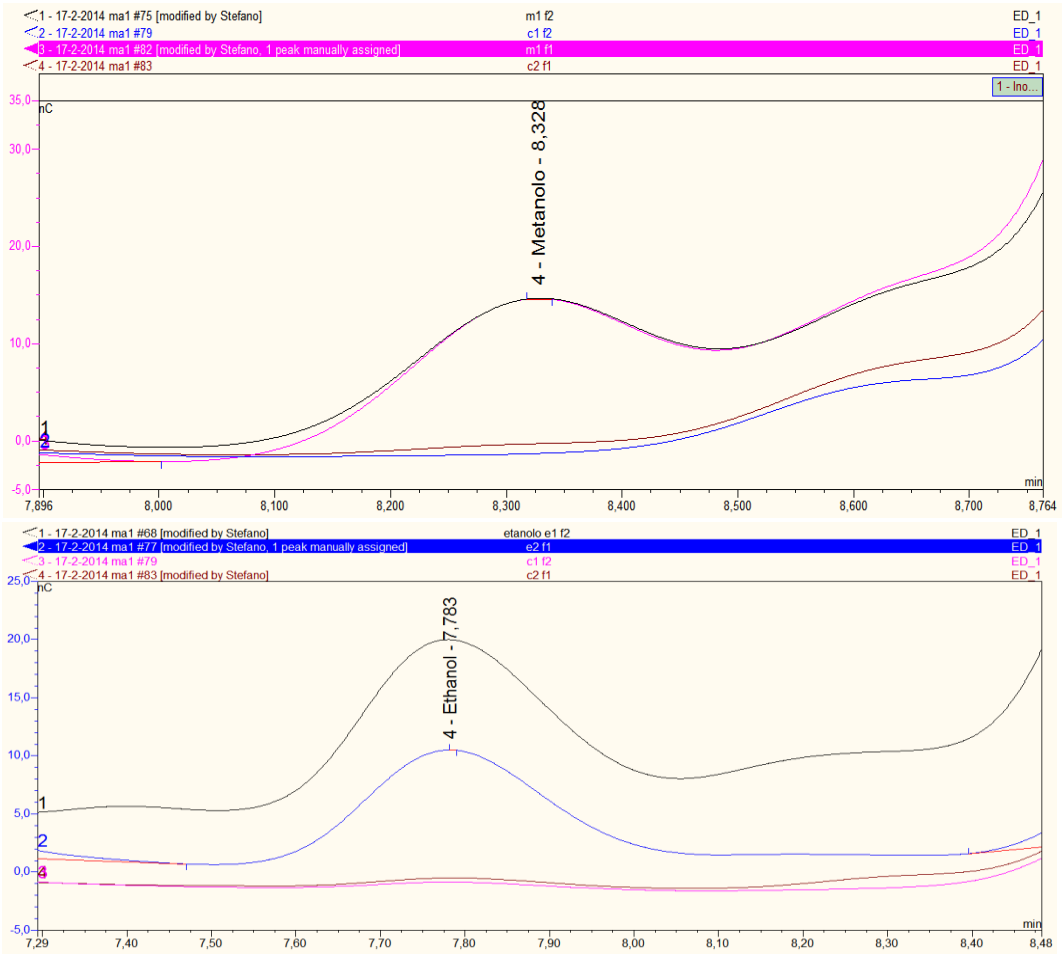

Figure 20. Accumulation of methanol fed with 5% (v/v) and ethanol fed 5% (v/v) in leaves of rocket

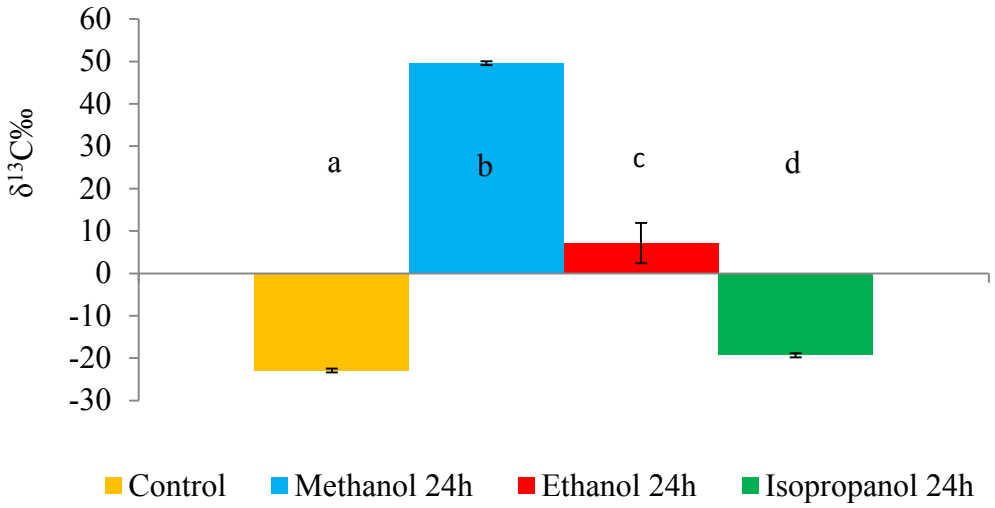

Figure 21. Emission of $^{13}CO_2$ from leaves of rocket exposed to different 15mM alcoholic solutions for 24 hours

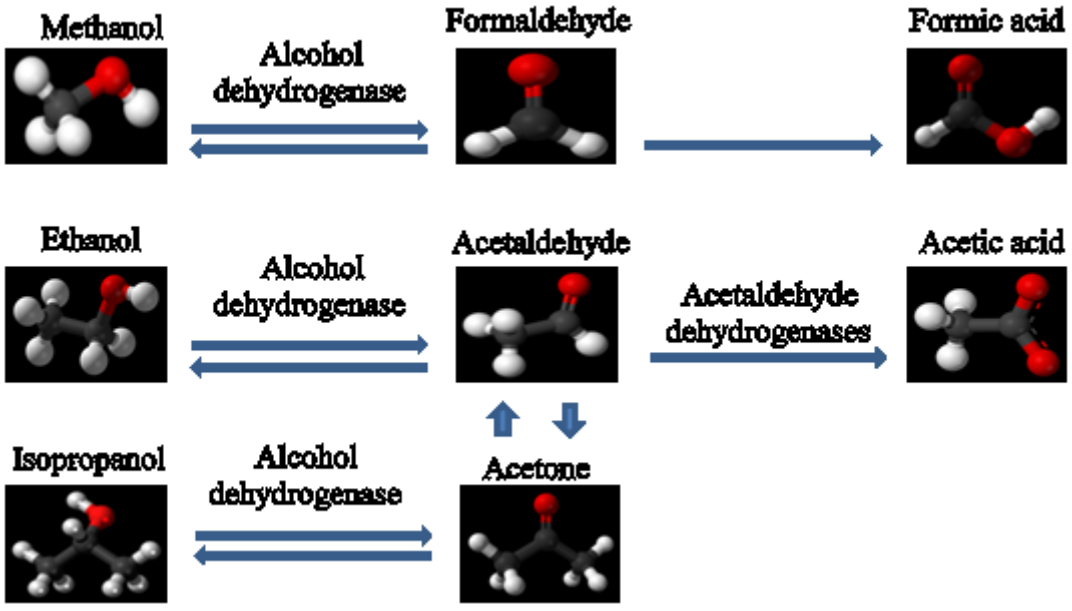

Figure 22. Schematic representation of methanol, ethanol, isopropanol, and their respective oxidized products of the metabolism

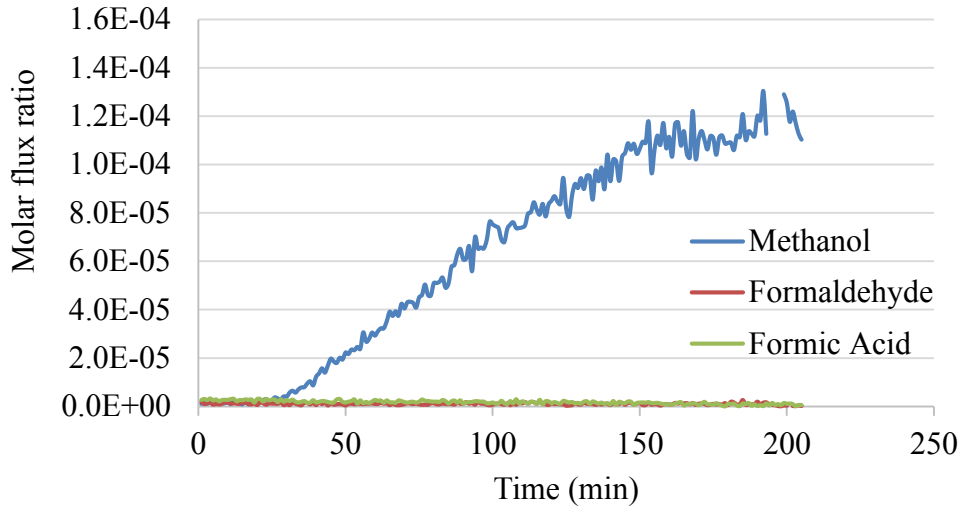

Figure 23. Molar flux ratio of methanol, formaldehyde and formic acid to water transpired by stomata before and after the feeding with 15mM methanol. Feeding started at minute 7th.

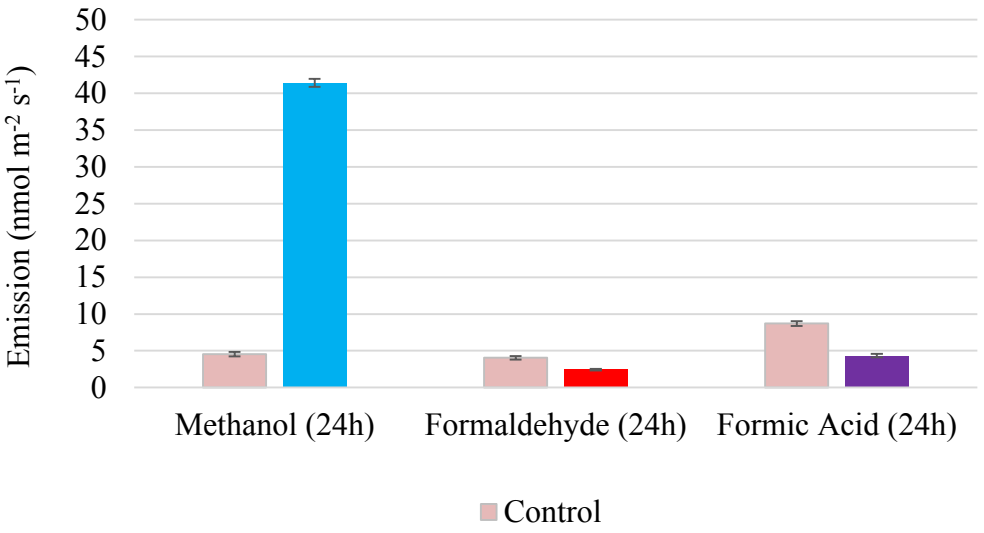

Figure 24. Emission rate of methanol, formaldehyde and formic acid 24 hours after the feeding with 15mM methanol

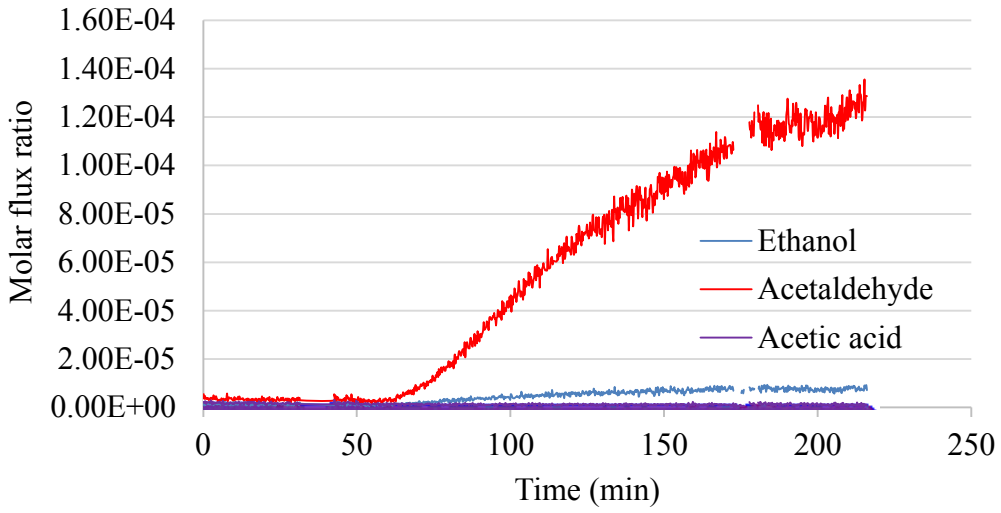

Figure 25. Molar flux ratio of ethanol, acetaldehyde and acetic acid to water transpired by stomata before and after the feeding with 15mM ethanol. Feeding started at minute 50th.

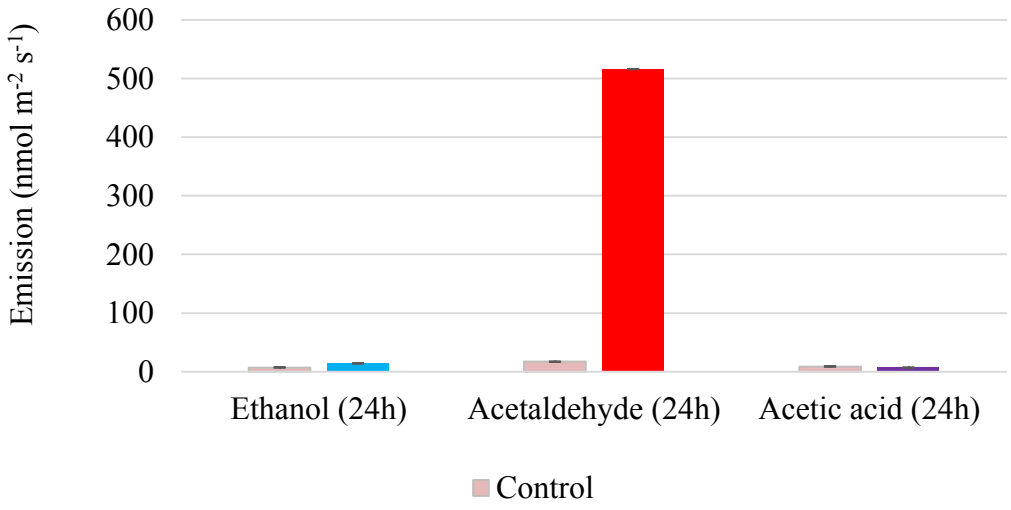

Figure 26. Emission rate of ethanol, acetaldehyde and acetic acid 24 hours after the feeding with 15mM ethanol

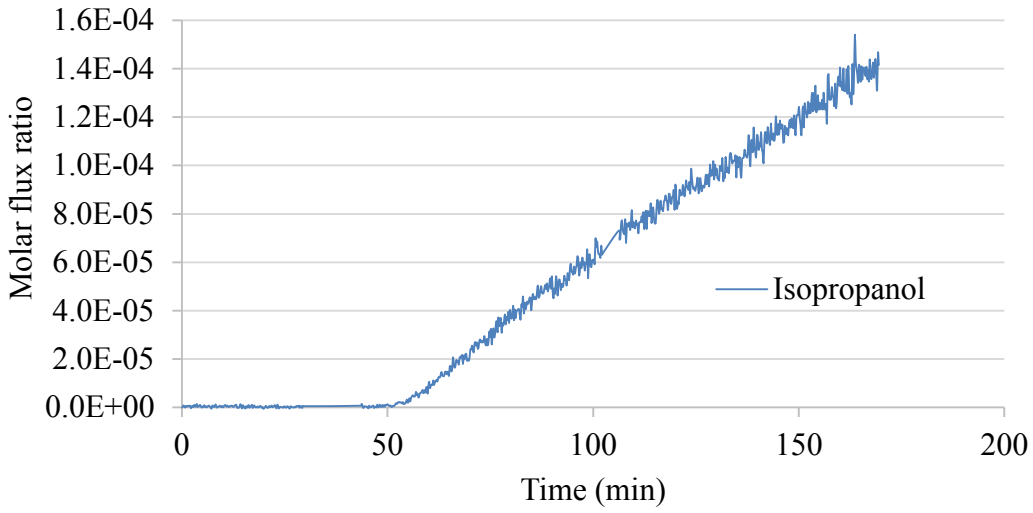

Figure 27. Molar flux ratio of isopropanol to water transpired by stomata before and after the feeding with 15mM isopropanol. Feeding started at minute 43rd.

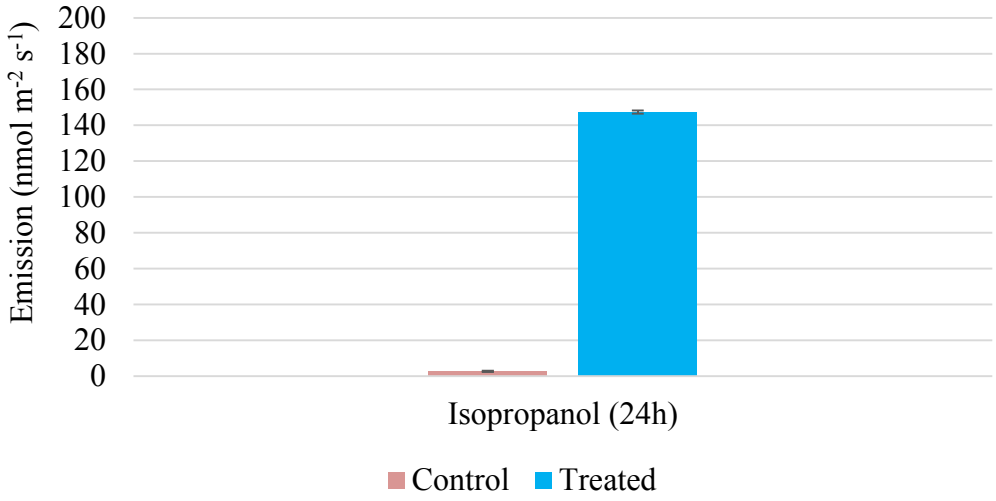

Figure 28. Emission rate of isopropanol 24 hours after the feeding with 15mM isopropanol

Effects of high CO_2 on physiology, biomass and quality of cardoon (*Cynara cardunculus* L.)

Introduction

There is a rising interests of consumers and industries on plant constituents that have a potentially high value in terms of nutraceuticals properties and as a feedstock for biorefinery activities. Dedicated cultivation systems to feed the nutraceutical and biorefinery industries are already gaining space on the market and are expected to increase in the future to sustain the development of the bioeconomy. In this respect knowing the environmental control of accumulation of metabolites relevant to define the quality of plant products for the nutraceutical and biorefinery industries would be of great importance to increase the efficiency of sustainable production systems. This would in turn increase the relevance of the agriculture sector and increase the incomes of farmers by providing them new and high value market sectors.

It is fully accepted that fibres have a relevant value as nutraceutical component of the diet, among them fructans and the fructo-oligosaccharides derived by the fructans partial hydrolysis are consider of high value (*99*, *100*). In the biorefinery industry fermentation is crucial to produce ethanol, and a vast number of high value molecules. The availability of fermentable sugars is then of major importance for the development of the bio-economy sector. Major site of accumulation of fermentable sugars in plants can be distinguished in a) non-structural carbohydrates (NSC); b) structural carbohydrate. While structural carbohydrate are polymer components of the plant cell walls and are difficult to depolymerize, NSC such as sucrose hexoses, starch, fructans raffinose and related oligomers, are either ready for fermentation or easily hydrolysable to monomeric fermentable components. NSC can have a storage role on many plant tissues. Cereal grains and potato tubers for example accumulate starch as main storage NSC, but fructans can also be accumulated to account for the main fraction of the dry matter in several plant families and tissues. Fructans are fructose-based oligo- and polysaccharides, with variable degree of polymerisation ranging from three to a few hundred units, can be linear (as inulin) or branched (*101*). Fructans accumulation is responsive to sucrose availability because sucrose is a substrate for fructans synthesis and can affect the expression fructans synthetic genes expression (*102*).

Photosynthesis is the processes involved in the reduction of carbon needed for growth, respiration and storage of reserves of plants. Sucrose has to be considered as the final product of photosynthesis in terms of reduced carbon availability for plant metabolism. Not only sucrose is the general form of reduced carbon transported from source (photosynthetic and storage organs) to sink tissues and organs, but at the cellular level, sucrose is central to carbon metabolism because it undergoes a futile cycle of hydrolysis and re-synthesis (*103*) that allow the cell to control the quantity of glucose6-P and fructose6-P. Phosphorylated hexoses are the starting point of practically all metabolic routes in the cell. High photosynthesis feeding on the sucrose and thereby hexose phosphate availability can favour non-structural carbohydrate accumulation. This has been show to occur in fructans synthesis in wheat (*102*).

Atmospheric carbon dioxide is the principal source of carbon for plants. Carbon dioxide enters leaves via stomata according to the difference in concentration between atmosphere and substomatal chamber. Therefore, an increase in the external CO_2 would determine and increase of concentration in the substomatal chamber. Hence, Rubisco would have more substrate for carrying out the Kalvin cycle and produce sucrose and starch, eventually. In fact, plants at ambient CO_2 do not operate at maximum capacity; they could increase the photosynthesis rate if they had more carbon dioxide available to process. Acclimation to higher atmospheric CO_2 would maximize the efficiency of photosynthesis increasing the quantity of reduced carbon for biomass production. However, increase in CO_2 concentration can trigger down-regulation phenomenon (*104*, *105*). To overcome this inconvenient, “sink” activity should be high.

Cardoon (*Cynara cardunculus* L is a fast growing plant of Mediterranean origin well known for its high inulin accumulation capacity (*53*). Since growth is highly energy demanding process, down-regulation of photosynthesis is unlikely during fast growth due to the strong “sink” effects of growth. The effects of high atmospheric CO_2 on fructans accumulation has been tested only in a few conditions on the synthesis and accumulation of inulin in cardoon has not been studied and its effects have only been described in a few species like wheat (*Triticum aestivum* L.)(*106*) and other C3 species (*107*). Therefore, the aim of this experiment was to study the effects of increasing atmospheric CO_2 availability up to 800ppm on i) occurrence of down regulation of photosynthesis ii) stimulation of increased photosynthetic product on fructans accumulation in roots of cardoon.

Material and methods

Plants of cardoon were grown in twin cabinets (Fitotron SGD170 Sanyo Gallenkamp UK) from seeds in floating system. Seeds were sown in styrofoam trays filled with sterile vermiculites. Each styrofoam tray was positioned in a plastic box preventively covered with black plastic film in order to prevent algae formation. For germination, just water was provided to seeds, soon after the germination, the water in every box was replaced with 8 litres of standard Hoagland solution 1.8 dS m^{-1} pH 6. Oxygenation of the nutrient solution was assured by air pumps. The two cabinets were set at different CO_2 concentration: 400 ppm and 800 ppm respectively. Two boxes were prepared for each treatment. . The photoperiod was set at 12 hours with 70% of air humidity, light intensity was 650 μ mol quanta m^{-2} s^{-1}. After germination, plants grew for 5 weeks and then six plants per treatments (3 per box) were randomly chosen and harvested for growth and qualitative analysis as described in General methods. Before the harvest, during the fifth week, non-destructive analysis like gas exchange measurements were carried out as described in General methods.

Results and discussion

Cardoon is an allogamous species and plants originated by seeds are genetically heterogeneous. Growth under the experimental conditions was faster than expected hence during the experiment heterogeneity among plants of the same treatment was higher than expected, probably due to mutual shading and conditioned air flow within the cabinets. For this reason and for the limited number of replicates available in the small space of the growth chamber, some of the results show large differences between treatments but no statistical significance. Even when statistics do not support conclusive statements data support a clear trend induced by high CO_2.

Photosynthesis and photosynthetic acclimation. In 5 weeks of growth at different CO_2 partial pressure caused a clear although limited acclimation of photosynthesis. Figure 29Figure 30 show the A vs ci curve of cardoon leaves measure after 5 weeks of growth at 400 and 800 ppm of CO_2. The curves are fully in agreement with the model of Farquhar et al 1980 (*108*). Photosynthesis increased linearly with CO_2 partial pressure at low sub-stomatal CO_2 concentration, as it would be expected being the assimilation rate strictly

determined by the activity of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (EC. 4.1.1.39). Maximum photosynthetic rate was particularly high for a C_3 plant showing the high assimilatory potential of the species and that the growing conditions were particularly favourable to cardoon. At all sub stomatal CO_2 partial pressures photosynthesis of the control leaves was slightly higher than that of the high CO_2 ones. This is a clear indication that growth at high CO_2 partial pressure caused an acclamatory down regulation of cardoon leaves that caused a reduced ability of cardoon photosynthesis to use high CO_2 partial pressure. Nevertheless, this reduction was limited and did not offset the increase of assimilation due to the higher CO_2 partial pressure present in the growth environment. This indicates that plants grown under high CO_2 partial pressure should have experienced an increase of photosynthetic products availability.

Leaf carbohydrate status and growth. The products of the Calvin cycle are triose phospates, that a) re-enter the Calvin cycle for the regeneration of the carboxylation substrate, b) are used for primary starch synthesis c) are exported to the cytoplasm for sucrose synthesis. Starch and sucrose content in leaves normally depends on the specie, on the phase of the diurnal cycle, on stress factors and many other internal and external effectors but are invariably positively affected by high photosynthetic rates and particularly by high photosynthesis/utilisation ratio; in other words, by a high source/sink ratio at the leaf and plant level. Leaves of plants grown in high CO_2 had significantly much higher sucrose, starch hexoses than leaves grown under normal CO_2 partial pressure. They also had an higher starch over sucrose ratio. These data clearly indicate that the higher photosynthetic rate in high CO_2 made photosynthetic products more available to the plant. The fact that starch increased more than sucrose in relative terms, indicates that photosynthesis of plants grown under high CO_2 was slightly sink limited, and this is in agreement with data on photosynthesis. The sink limitation indicates that cardoon was only partially able to use the increased photosynthate availability to sustain faster growth. In real terms, the increase of leaf dry matter can largely be accounted by the increase of their non-structural carbohydrates (data not shown). Furthermore, unclear sign of an increased root growth was obtained. Since no statistically significant changes in the leaf area were recorded the increased photosynthate availability was, up to this stage, only due to the increase of photosynthesis per unit leaf area due to high CO_2.

Fructans accumulation in roots. Fructans can play several functions in plants but in cardoons the primary function of fructans accumulation in roots is storage. Fructans are stored in root of cardoon up to 60-70% of their dry weight with a clear seasonal dynamic and are re-used to sustain sprouting after the summer drought period (*53*). In this experiment cardoon plants were forced to accumulate fructans by increasing their source/sink ratio. As expected, this increased the availability of leaf non–structural carbohydrates, notably sucrose and starch. The analysis of tap roots and fine roots of cardoon plant reveal that fructans were already accumulating in the tap roots while only minor content was found in the fine roots. Tap roots of plants grown under high CO_2 had a slightly higher content of fructans although it was not statistically significant.

Conclusions

Cardoon grew very fast in controlled environment and photosynthesis took advantage of high CO_2 partial pressure with limited down regulation by reduced sink capacity. High photosynthesis was sufficient to cause an increase of leaf non-structural carbohydrates. In the time of the experiment, indications of a feedforward action of high photosynthate availability on growth and fructans accumulation were recorded, but the high variability of the plant material, the limited time duration of the trials and the limited number of replicates did not allow to have statistically significant results on the stimulation of fructans accumulation in high CO_2. However, it is particularly relevant that a) cardoon can grow very fast under fully controlled conditions; b) fructans under optimal growth conditions can start accumulate as soon as few weeks after plant emergence, c) high CO_2 can very likely increase growth and fructans accumulation in tap roots of cardoon but this would require longer than 5 weeks to be relevant in quantitative terms. Further understanding in the regulation of fructans accumulation in plants would be of use in the long term for the biomass production dedicated to the biorefinery industry that would be necessarily produced in the open fields. However the nutraceutical and pharmaceutical industries are very interested in plant biomass for the production of health related preparations that require very strict control on plant growth conditions to ensure safety of their products. Results from this experiment point to the possibility to use cardoons for fructans and fructo-oligosaccharides production in fully controlled conditions in order to increase quality of the biomass feedstock.

Tables and Figures

Table 19. Growth analysis of plants of cardoon acclimated to different concentrations of CO_2

Treatment	Root DW (g plant^{-1})	Leaf FW (g plant^{-1})	Leaf DW (g plant^{-1})	Leaf surface (cm^2 plant^{-1})	SLDW (mg cm^{-2})
400 ppm	2.27±0.67	177.4±7	12.99±0.3	2694±82	4.83±0.22
800 ppm	2.79±0.32	204.1±14	16.21±0.9	2878±222	5.70±0.31
Significance[a]	ns	*	*	ns	ns

*ans, *, Nonsignificant or significant at $P < 0.05$, respectively.*

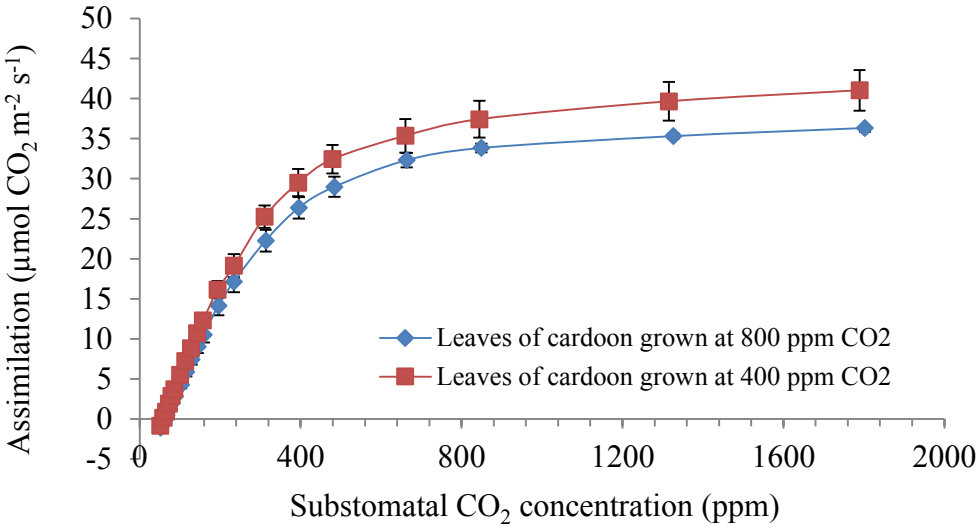

Figure 29. Assimilation vs sub-stomatal CO_2 concentration in leaves of cardoon acclimated to different environmental carbon dioxide concentration.

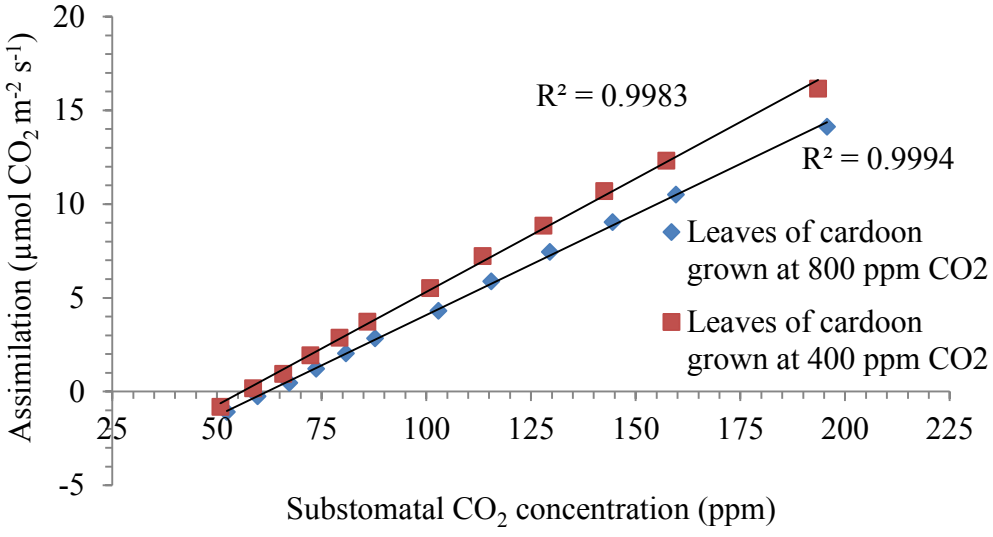

Figure 30. Rubisco activity related to the slope of the curve in leaves of cardoon acclimated to different environmental carbon dioxide concentration

Table 20. Carbohydrate content in leaves of cardoon acclimated to different ambient CO_2 concentrations

Treatment	Sucrose (mg/g FW)	Starch (mg/g FW)	Starch/ Sucrose	Total soluble Carbohydrate (mg/g FW)	Total Carbohydrate (mg/g FW)
400 ppm	4.4±1.5	3.1±1.3	0.66±0.10	5.20±1.70	8.29±2.94
800 ppm	15.8±3.2	18.1±4.3	1.29±0.23	21.33±3.41	39.39±6.31
Significance[a]	*	**	*	**	**

[a]*ns, *, Nonsignificant or significant at $P < 0.05$, respectively.*

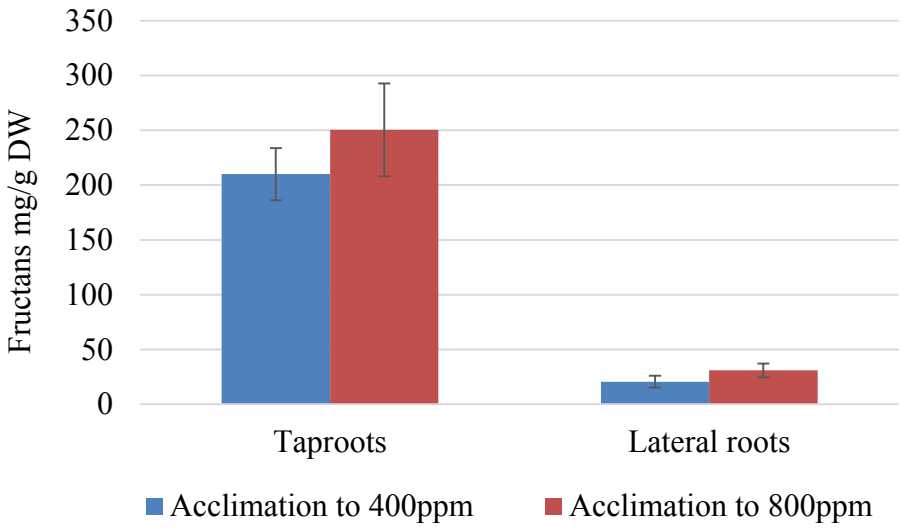

Figure 31. Content of fructans in taproots and lateral roots of cardoon acclimated to different ambient CO_2 concentrations

General Conclusions

The quality of vegetables and fruits depends on the interaction of several factors: genetic, agronomical practices and environmental conditions. They affect differently both qualitative and organoleptic features in the short- and long-term adaptation. Finding the best combination of those factors is the challenge that growers have to face in order to meet high quality standards. The choice of the best genotype is a fundamental step to take for producing high nutritional value food. Green revolution has sought to develop new hybrid vegetable cultivars enhancing yield, taste appearance, disease tolerance and shelf life (*109*), lycopene and content of other carotenoids in tomatoes (*110*), flavonols and phenolic acids in lettuce (*111*), glucosinolates and carotenoids in European Brassiacaceae, e.g. broccoli and cauliflower (*112*). Specific glucosinolates have also been found responsible for the bitter taste of rocket (*113*). However, agronomical practices like soilless culture, irrigation and fertilization can permit to save energy and resources while maintaining good productions. The synthesis of health-promoting compounds can be promoted as well as the total antioxidant activity, by increasing the conductivity in the nutrient solution in sweet pepper, cucumber, eggplant, celery and watermelon (*32*). Pondered water stress induced by irrigation regimes can contribute to the control the vegetative growth of plants and the improvement of fruit quality (*114*); fertilization can have profound effect on the final product in terms of nutritional and organoleptic properties, accumulation of antioxidant compounds (*115*).

On the other hand, plant food quality can even be affected negatively when those means are not applied consciously because the accumulation of undesired compounds like nitrates, can be detrimental for human health. In fact, nitrates can be converted into nitrites by commensal bacteria starting from the mouth; the conversion efficiency in human body changes according to age and stomach pH: infants and patients with gastroenteritis have higher rate of conversion (*76*). Nitrites oxidize the ferrous iron the haemoglobin to ferric form producing methemoglobin which cannot bind to oxygen (*116*). For example different cultivars of lettuce accumulate nitrates in leaves in different concentrations. Leafy vegetables with nitrate concentrations exceeding European thresholds are not allowed in EU market. Distinctions are made according the season of production: in winter time higher concentrations are allowed (Table 21) due to the dependence of the enzyme nitrate reductase (EC 1.7.1.1) on light availability (*27*). Light, among the environmental factors that can affect fruit and vegetable quality, requires particular

attention, specifically in the case of nitrates. In "Transparent photovoltaic panels as shading tool in sunny areas" and "Zeolite Potential Application in life Support", the tendency to accumulate nitrates, especially in leafy vegetables, was evident when plants were grown under low light intensity. For example, leaves of rocket grown in shaded conditions (PVPs and net) showed higher content of nitrates in comparison to control (Table 8). In that specific case, control plants adapted to a double intensity of light in comparison with shaded plants which averaged 700 ppm against 130 ppm measured in control plants. In spinach, the difference between treatments was lower than in rocket (Table 9). However, even the highest value is still in compliance with the European regulation.

Table 21. Maximum levels for nitrates in foodstuffs (29)

Foodstuffs	Maximum levels (mg NO_3/kg)	
Fresh spinach, (*Spinacea oleracea*)		3500
Preserved, deep-frozen or frozen spinach		2000
Fresh lettuce (*Lactuca sativa* L.) (protected and open-grown lettuce) excluding lettuce listed in point	Harvested 1 October to 31 March Lettuce grown under cover Lettuce grown in the open air Harvested 1 April to 30 September Lettuce grown under cover Lettuce grown in the open air	 5000 4000 4000 3000
"Icerberg" type lettuce	Lettuce grown under cover Lettuce grown in the open air	2500 2000
Rucola (*Eruca sativa*, *Diplotaxis* sp., *Brassica tenuifolia*, *Sisymbrium tenuifolium*)	Harvested 1 October to 31 March Harvested 1 October to 31 March	7000 6000
Processed cereal-based foods and baby foods for infants and young children		200

The same species was also used for the assessment of the possible effects of different zeolites on the quality of vegetables. Regardless the effects of treatments, which were non-significant in many of the analysis performed, very high concentration of nitrates

was found in leaves. In that case, plants grew at 65 μE m^{-2} s^{-1} (a light intensity found to be slightly above the compensation point for rocket in other trials, data not shown) that was approximately five times lower than the incident light received by rocket plants adapted to shade conditions in "Transparent photovoltaic panels as shading tool in sunny areas". Low light contributed to increase nitrate content in leaves by almost ten times. Certainly, a straightforward comparison between the two experiments is to be considered carefully but it is conceivable to state that light intensity played important role in the activation of the enzyme nitrate reductase and the consequent accumulation of nitrates in edible tissues. Since rocket has been listed among the regulated foodstuffs for its capacity to accumulate relevant amounts of nitrates in leaves, those plants would not have been probably in compliance with the European regulation. Moreover, high consumption of that food could have put at risk consumer's health.

Low light exposure for vegetables may have negative effects also on the synthesis and accumulation of desired nutritional and nutraceutical compounds that are fundamental for maintaining the health of a human body. Tomato fruits of plants grown in PG exhibited lower total carbohydrates content than control plants Table 11. Tomato plants grown in fully controlled environment produced fruits with slightly lower carbohydrate content when exposed to low incident light Table 7. The yield of tomato per plant was not affected by adaptation to different light intensities in both cabinet and prototype cultivation, but differences are evident if yield in fully controlled environment is compared with the yield in greenhouse prototypes: control plants produced 19.2 g $plant^{-1}$ in the cabinet and 8.55 g $plant^{-1}$ in the CG. The maximum photosynthetic capacity was lower in plants grown in greenhouse prototypes although the incident light was similar to that provided in the cabinet. Perhaps, light was not the major driver for yield between the two environments so if plant food production is to be enhanced, other factors should be kept under control in order to avoid that discrepancy. Minor differences were observed in the lycopene content among the treatments and similar concentrations were found in fruits produced in cabinet and greenhouse prototypes. Lycopene is a natural carotenoid with high nutraceutical value because its high antioxidant activity (*117*). Controlling the environment where fruits and vegetables grow is an important prerogative for future plant food production because yield and quality can be dramatically reduced by uncontrolled factors. For example, low content of lycopene in fruits of tomato is usually associated with an increasing the in the surface temperature of the fruit (*118*). However, high

temperature can be reached during high light intensity exposure but high light intensity is also a condition of high energy availability for photosynthesis and then, growth. Therefore, a middle ground for both environmental factors is needed and this requires further investigations on the matter. So far, these investigations have demonstrated that high solar radiation can be reduced by using PVPs instead of conventional nets without affecting negatively both yield and quality in rocket, spinach and tomato in a given controlled environment. However, tomato yield was considerably reduced when grown in greenhouse prototypes where other uncontrolled environmental factors could not be regulated as precisely as in the cabinet.

Although light is very effective in influencing plant metabolism, there are other environmental factors that can promote the metabolism of desired compounds. Availability of CO_2 for plants in the atmosphere represents a limiting factor for the vital process of photosynthesis where inorganic carbon is reduced into organic forms, like cabohydrates, that are largely involved in transportation and accumulation of energy, signalling and mechanical purposes. The accumulation of specific carbohydrate class during normal growth in dedicated organs of the plant also represents an adaptive strategy to unfavourable seasons where drought or cold could be harmful for the plant (*99*). In *Asteraceae* family for example, accumulation of inulin in roots has been found to be the main reserve in both cultivated and wild species (*53*). Inulin, as all fructans, is considered a functional food. It is part of the human diet, it is available in many plants that we normally feed on like onion, leek and artichoke. It is a soluble fibre that cannot be digested by humans. Roots of chicory are among the primary sources of inulin on an industrial scale (*119*), moreover inulin is the primary additive form of fructans that is used the food industry (*99*). Nevertheless chicory is not the only *Asteraceae* that can accumulate considerable amount of fructans in roots. In the present study, the cultivation of cardoon (*Asteraceae*) in fully controlled environment at high CO_2 atmospheric concentration, has demonstrated that the synthesis of carbohydrates in leaves was fostered by high availability of the substrate (CO_2) for RuBisCo activity, and indications for a possible role of high CO2 in promoting the accumulation of fructans in taproots Figure 31 was obtained. Moreover, the phenomenon of down-regulation was only minimal in cardoon as possible confirmation of the initial hypothesis that fast growing species could cope better than others with high source of reduced carbon. The possibility to increase carbohydrate content in plant tissues, as demonstrated in cardoon, would also represent a

possible future strategy to offset fossil fuels for energy production. In fact, biomasses can represent a valid source of green energy in the next decades being fermentable sugars a valid substrate for bioethanol production and for the production of chemicals via fermentation. It is conceivable to argue that the production of biofuels can fuel the debate about food security and sustainability along with ethical concern regarding the possibility of competition for food production, increase of deforestation and other kind of land degradation (*120*). Because of that, competition for land use destination may arise. For example, recent investments in biofuel in United States have reduce the overall importation of oil from abroad but such strategy has also reduced supplies of food and grains with arguable reduction in the greenhouse emission (*121*). It has been estimated that we are currently exploiting half of the usable land in the world for agricultural and pastoral activities (*122*), further cultivation for biomass production would in turn determine a change in the land use destination of either marginal areas or woody lands with unavoidable soil degradation. Regardless the increasing demand for new lands that would come into being if humankind is to rely on biomass energy in the next future, 70 million ha are due to be subtracted to natural ecosystems in developing countries in order to meet the increasing demand for food by 2050 (*6*). Increasing the quality of the biomass can make production systems more efficient and hence reduce the need of land for biomass production. However, it is conceivable that agricultural biomass production for industry in the future would be devoted to the production of high value compounds as nutraceuticals and pharmaceutical preparations, and that low value biofuels would be mainly produced by recycling waste biomass.

Cropping in controlled environments could help reduce the impact of agriculture on terrestrial environment but in manned space applications, it will be the only possible solution for food production via BLSS. Since replenishments from earth of food and water supplies could be difficult or prohibitive, recycling biomass and water will not be an option. Recycling water and biomass could represent a threat for living organisms in a BLSS if the accumulation of pollutants is excessive. In BLSS food safety and food security are the most important prerogatives for astronauts. Starting from the list of organic and inorganic pollutants dissolved in the CLC, the effects of MEI were tested on rocket for inhibitory effect on the germinability of seeds and physiological response in 5 weeks old plants fed in the short period with alcoholic solutions. Results show that the concentration of MEI found the in the CLC was too low to compromise the germinability

of rocket seeds. However, an increase in the concentration of ethanol in CLC can affect negatively the germinability: concentrations higher than 0.2% (v/v) have inhibitory effects on seeds of rocket. MEI can be up taken by roots and translocated to leaves in few minutes. Detectable accumulation of MEI in edible tissues is possible if feeding at the petiole level is performed using either 5% (v/v) methanol or ethanol. Isopropanol was not detected by ionic chromatographer in leaves. However, isopropanol is transported towards leaves if feeding is applied at root level and on-line PTR-MS measurements revealed important emissions of such alcohol from stomata 10-15 minutes after feeding. Once in leaves, MEI can be released in the atmosphere through transpiration either as alcohol, in the case of feeding with methanol and isopropanol, or as product of oxidation (acetaldehyde) as found when feeding via nutrient solution with ethanol was applied. Emission of oxVOCs by plants has been known for a while and it is present in rocket as well. This was also demonstrated by the data collected before the feeding. Figure 24, Figure 26 and Figure 28 show that MEI and their relative metabolites are normally emitted by rocket leaves even in non-stressed conditions. MEI emission rates can increase remarkably after feeding from the substrate. Therefore, the concentration in the atmosphere of some pollutants in a BLSS can increase if the plants are fed with CLC, as consequence of the intrinsic oxidative metabolism for alcohols. Ethanol was quickly oxidized to acetaldehyde whom was released in the atmosphere via stomata transpiration in a matter of minutes. Emission of acetaldehyde was still active 24 hours after the feeding, though occurring at lower concentration. Interestingly, methanol, who was not mainly emitted as either formaldehyde or formic acid (products of its oxidative metabolism), produced higher $^{13}C/^{12}C$ in respiration. This sheds light on the different affinity of the rocket metabolism in scavenging MEI. The AHD is the enzyme that mediates the oxidation of an alcohol into its oxidized metabolite. Since ethanol was promptly oxidized to acetaldehyde, it can be speculated that AHD was already present and active in leaves of rocket at the moment of feeding. Its activity can increase over time since the emission rate of acetaldehyde 24 hours after the feeding was still high and the emission of ethanol remained low, although the concentration of ethanol in the solution had decreased during the 24 hours following the feeding (data not available). Therefore, the emission rates of undesired compounds as acetaldehyde that is a possible carcinogenic molecule (*123*) can increase over time if ADH activity is enhanced but the confirmation of this hypothesis requires deeper analysis. However, according to our results, the use of

CLC for plant cultivation in BLSS should not pose a threat for the crew since negative effects were found neither in germinability nor accumulation of oxVOCs in plant food.

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