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EXPERIMENT INVESTIGATING THE INFLUENCE OF OXYGEN DEFICIENCY ON PLANTS GROWN IN MICROGRAVITY

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Abstract
Plant growth experiments in the Bulgarian developed SVET Space Greenhouse onboard the MIR Orbital Station proved that precise monitoring and control of the substrate moisture levels is not enough to provide adequate moisture for plant roots because the water distribution in microgravity differs significantly from the one on Earth. Microgravity changes the behavior of fluids and gases, disturbs the water-air balance in the substrate medium and causes difficulties in providing even substrate moisture in the whole volume. As a consequence excessive irrigation may occur and the high water content may cause oxygen deficiency in the plant root area. Some ground-based biotechnology experiments were carried out to improve the scientific knowledge of water distribution in the root substrate media and its impact on plants. A space experiment with Arabidopsis thaliana plants grown in the ISS European Modular Cultivation System, with the objective to investigate plant response to oxygen deficiency occurring in microgravity in the process of substrate moisture control, is planned. Plant growth and development, the ultrastructure of cell organelles in plant roots and leaves, the activity of specific marker enzymes and cytosolic Ca^{2+} concentration in plants grown under both microgravity and synchronous ground - based 1-g conditions will be investigated.

Background

Development of a Bioregenerative Life Support System is an important area of research motivated by the need to support crew life during future long term human spaceflights. Such a system, based on plants, can provide food and clear the air like the natural biological exchange of chemical elements in the earth biosphere. In addition plant crops grown onboard have a significant effect on the crew emotional frame creating psychological comfort for the astronauts during their long isolation. Research work on the SVET project to study the ways and technology of plant growth and utilization in space was an effective step towards the development of such a system.

The SVET Space Greenhouse (SG), developed in the Space Biotechnology Department at the Space Research Institute, Bulgarian Academy of Sciences to investigate plant growth in microgravity was launched onboard the Kristall module under a Bulgarian-Russian (IMBP, Moscow) project and was regular equipment onboard the MIR Orbital Station (OS) since 1990 when the first vegetable plant experiments were carried out (Ivanova et al, 1993). Second modification SVET-2 SG equipment with considerably improved technical characteristics of separate units was developed and launched onboard

the MIR OS in 1996 under NASA financial support. An American Gas Exchange Measurement System (GEMS) was developed in the Space Dynamics Laboratory at Utah State University and added to the Bulgarian equipment to monitor more environmental parameters (Ivanova et al,1998).

A series of long-term plant experiments was carried out in the SVET-GEMS complex by different international scientific teams onboard the MIR OS during the period 1996-2000. Significant results in the field of Gravitational Plant Physiology were obtained in these experiments. Second-generation *Brassica Rapa* and *Apogee* wheat seeds were produced under microgravity conditions and reiteration of the seed-to-seed plant cycle was achieved (Musgrave et al, 2000, Levinskikh et al, 2001). The SVET SG experiments improved significantly our understanding of the physics of water and oxygen movement in the substrate media in space. Although a lot of environmental factors have an effect on plant development none of them have such restricting influence as the water, air and nutritive control in the root area. The experiments proved that precise monitoring and control of the substrate moisture is not enough to provide adequate and uniformly distributed water and oxygen content in the root module volume which is critical to normal plant growth. Microgravity disturbs the water-air balance in the substrate medium and causes difficulties in providing even substrate moisture in the whole volume. As a consequence excessive irrigation may occur and the high water content may cause oxygen deficiency in the plant root area. This calls for a wide program of investigations to fully study and master the processes going in an artificial media for plant growing.

Scientific grounds for the investigations

The water movement in microgravity differs significantly from the one on Earth. Microgravity changes the behavior of fluids and gases - the water distribution in the substrate medium is driven by capillary forces and the gases move in a diffusion way. The quantity of the air in the substrate medium is in inverse proportion to the quantity of the water containing there. On Earth the water-air balance is controlled by gravitational forces. Moving in the substrate volume water completely fills the air pores of the substrate expelling air from there and the opposite – going out it clears them for the air. In this way the process of aeration proceeds normally.

Under microgravity water fills the inner capillaries of the substrate particles and forms water film on the particle surface. Each additionally supplied water quantity tends to close the aeration pores in the narrow sections trapping the air and forming bubbles thus breaking the air conductivity. The air pathways breaking down can lasts from hours to days depending on the intensity of water consumption by plants and leads to oxygen deficiency (Bingham et al, 2000).

On the other hand the control of the systems for water supply meets difficulties in providing even substrate moisture in the whole volume. In microgravity this can lead to excessive irrigation and the high water content may cause oxygen deficiency. Symptoms of oxygen deficiency stress - induction of fermentative enzyme activity in roots and accumulation of carbohydrates in shoots were found in different plants grown in different space facilities (Porterfield et al, 2000; Stout et al, 2001). At present, more information is still needed to quantify growth reduction due to oxygen deficiency.

The plant metabolism characteristics are changed under low oxygen availability conditions. Higher plants are aerobic organisms and supply of molecular oxygen from their environment is critical for supporting respiration and other life-sustaining oxygenation reactions. In low oxygen availability (hypoxia) and less frequently in total absence of oxygen (anoxia) the oxygen-dependent pathways, especially the energy-generating systems in plant tissues are totally suppressed. The functional relationship between roots

and shoots is disturbed and both carbon assimilation and photosynthetic products utilization are suppressed.

It has been suggested that in plants grown under microgravity an oxygen deficiency could occur as a result of microgravity-induced changes in fluid and gas distribution in the surrounding environment (Stout et al., 2001).

Mitochondrial respiration is greatly affected by the low oxygen availability. In the absence of oxygen as a final electron acceptor in mitochondrial respiration chains the pyridine nucleotides reduced in glycolysis and in the Krebs cycle cannot be deoxidized. As a consequence of this ATP, which is the major energy source in cell metabolism, is not produced, thus leading to a switch to a fermentative metabolism allowing both the reoxidation of NADH and ATP. Three main metabolic pathways are active under oxygen deficiency conditions – alcoholic, lactic acid fermentation and a species-specific metabolic pathway in which alanine is produced from glutamine and pyruvate catalyzed by alanine transferase (Perata et al., 1993). There are several operating pathways in NADH reoxidation (Crawford, 1978) but the fermentation generating lactate and ethanol plays a major role in the anaerobic recycling of pyrimidine nucleotides. Lactate is produced by the action of lactate dehydrogenase while ethanol production is catalyzed by alcohol dehydrogenase.

There are several ways of regulating the anaerobic metabolism (Davies, 1974). In normal oxygen availability the cytoplasmic pH is higher than neutral. In early phases of anaerobiosis lactate production prevails resulting in a pH decrease and activation of enzymes with acid pH optima. Lactate dehydrogenase is inhibited while pyruvate decarboxylase and alcohol dehydrogenase are activated. The changes in cytoplasmic pH are assumed to regulate the metabolic switch to alcoholic and lactic acid fermentation. The ethanol production following the physiological drop of cytoplasmic pH is the main fermentative process (Fox et al., 1995). Some data in literature suggest that the pH drop has been caused not only by metabolic activity but also due to the action of H+-ATPases (Saint-Ges et al., 1991). Cytoplasmic acidification can lead to cell death caused by the reduced glycolytic flow (due to the lower pH level), disturbance of the internal cellular compartments and increased ATP hydrolysis rate due to acid phosphatases activation.

Gene expression is strongly altered under conditions of oxygen deficiency. A set of about 20 new polypeptides is induced. They are anaerobic proteins participating in the starch metabolic pathways of glycolysis and alcohol fermentation (Sachs et al., 1980). An induction of two genes not related to sucrose metabolism has been observed. According to some hypotheses they are responsible for the aerenchyma formation (Sachs et al., 1996). Aerenchyma is a highly porous plant tissue, which has internally interconnected gas-filled spaces allowing root aeration as an important adaptation to hypoxia and anoxia. The metabolic plant response to oxygen deficiency includes three steps. The first one is the rapid induction of the signal transduction chain components. There is evidence showing that cytosol Ca^{2+} can mediate the oxygen signal transduction pathway and that the rapid increase of cytosol Ca concentration is due to the release of Ca^{2+} from the endoplasmic reticulum and mitochondria (Subbaiah et al., 1998). The signal perception activates the second step – the metabolic adaptation in which genes involved in the glycolytic and fermentative pathways are up-regulated. The third step includes aerenchyma formation.

Considerable changes in mitochondrial structure are observed under conditions of hypoxia and anoxia. Chloroplast membranes integrity is destroyed and the content of starch grains is increased. The ribosomes do not form polysomes and are predominantly single, the number of dictiosomes as well as the number of micro-bodies are reduced (Bailey–Serres Y., Freeling, M., 1990).

Some ground-based and space biotechnological experiments and plant response investigations are planned to be carried out with the objective to improve the scientific knowledge of water and oxygen distribution in the root substrate media in microgravity.

Ground-based experiments

Although it is impossible to repeat on Earth the pattern of water distribution in the substrate medium in microgravity some separate events could be imitated. The excessive water levels and the unfavorable aeration status of the substrate medium could be replaced on Earth by waterlogging process.

Three ground-based experiments were conducted recently in the laboratory prototype of the SVET-2 SG to study influence of oxygen deficiency on plant growth parameters. The objectives of the experiments were to study the impact of changes in substrate moisture levels and consequent waterlogging on height, phonological characteristics and physiological response (plant growth parameters, photosynthesis, etc.) of pea plants (*Pisum sativum L. cv "Ran-1"*) grown in substrate Balkanine with 1,0 – 1,5 mm particle size.

During **Experiment 1** (4 March – 3 May 2004) pea plants were grown initially at optimal substrate moisture levels (1/3 from saturation), a small rise of the substrate moisture (5% for 24 hours) was applied demanded by the growing plants and then the substrate was completely flooded. We observed that the small rise of the substrate moisture led to leaf wither and drop before the waterlogging stress was applied. Plants grown in waterlogging conditions showed typical visual signs of oxygen deficiency stress. Pea plants stopped to develop in height and died. On the 3rd day after the waterlogging treatment chlorosis was observed on plant leaves. The leaf wither and senescence continued during the treatment and plants died before reaching maturity. On the 10th day of the waterlogging stress new shoot growth was observed from the stems of the withered plants.

The design of **Experiment 2** (8 July - 6 August 2004) allowed monitoring of the moisture dynamics vertically in the substrate volume during initial substrate moistening, initial plant growth, 5% moisture rise for 24 hours and subsequent recovery to the previous substrate moisture level. The experiment revealed that the moisture control during the process of initial moistening of air-dry substrate Balkanine led to saturation of the bottom substrate layers in the RM. It was also found out that even a small raise of moisture in the substrate volume before the substrate moisture level necessary to achieve good water conductivity was reached, leads to moisture increase in the zone where roots are developed at this moment. Although for a short time the higher water content in that zone has obviously caused the leaf wither and drop.

During **Experiment 3** (3 May – 6 June 2005) physiological response of pea plants was studied in the same substrate moisture dynamics as in Experiment 2. Observations were made 24 h before the moisture was risen (control measurement) and 24 h, 72 h and 144 h after the rise. The moisture rise had a slight effect on growth parameters – Dry / Fresh Weight Ratio, Relative Growth Rate (RGR) and Shoot Elongation Rate (SER). The net photosynthetic rate (Pn) declined with the substrate moisture rise, while the dark respiration rate (Rn) increased, and the chlorophyll content of the leaves did not change during the experiment (Ilieva I. et al, 2005).

The results obtained showed that even a small rise of moisture during plant growth in substrate when saturation had occurred in the bottom layers can lead to plant oxygen deficiency response and to reduction of the photosynthesis. The experimental work is continuing and the new results from the next Experiment 4, carried out with lettuce plants in March-April 2006, are reported on this conference.

Space experiment description

The European Space Agency SURE Project provides new opportunities to use the microgravity facilities onboard the International Space Station (ISS) for plant growth research. An experiment with *Arabidopsis thaliana* plants grown in the European Modular Cultivation System (EMCS) of the US Lab is planned, with the objective to investigate the microgravity-induced hypoxia by exploring the physiological and biochemical response of the plants.

The experiment biological program requires two synchronous (space and ground-based) experiments to be carried out with identical equipment, technical support accessories and under similar environmental conditions. The Bulgarian developed SVET-M experimental equipment will be mounted in the EMCS Experiment Container (EC) of one of the two centrifuges and will operate at 0-g. The experiment hardware is a Root Module (RM) of 60x60x60 mm external size, made by Plexiglas, Lexan, or other similar transparent material and 5 mm in thickness. RM is filled with substrate Balkanine which represents 1,0-1,5 mm particles of natural zeolite, pre-charged with nutritive. A watering system is mounted in the substrate volume. It goes out of the RM and is connected with the EMCS Water Supply System Reservoir. The planting surface has 4 holes in which the seeds are planted and fixed before equipment packing prior to launch.

For normal experiment execution the EMSC Atmospheric Control System will provide the following environmental parameters for the SVET-M unit:

- air temperature range of +21^{o}C, +/-1^{o}C;
- air relative humidity range of 60-75%;
- air gas composition – CO_2 0.035 kPa;
- doze water injections in accordance with preliminary appointed program;
- lighting of 50 W/m^2 PAR and 16/8 hours light/dark cycle; continuous (24h) illumination is preferable;

Photo pictures will be taken on preliminary appointed time intervals and downloaded from ISS daily to determine the plant growth rate. All the data about the environmental parameters maintained in SVET-M EC will be sent daily to the Bulgarian scientists for assessing the onboard experiment and carrying out the synchronous ground-based experiment.

Plant sampling at approximately the 30th day of the experiment will be made. All the seedlings except one will be cut and samples will be stored in a freezer at –20^{o}C. One of them (2-3 leaves) will be cut up, fixed in fixation solution and stored at a temperature of –4^{o}C till the time it will be returned to Earth.

In case of long-duration experiment when a “seed to seed” cycle is achieved and seed pods are developed they will be harvested at the 60th day of the experiment and stored under the conditions required for equipment stowage and transportation (4 to 10^{o}C temperatures and dry air) till their return to Earth. All the biological samples returned to Earth will be delivered to the scientific research team for analysis.

Experimental methods and expected results

The following analyses of the experiment plant samples returned after 30(60) days will be performed:

- Determination of the changes in the cytosolic concentration of Ca^{2+} and assessment of their role in controlling the physiological response of plants to low oxygen availability.
- Determination of alcohol dehydrogenase and lactate dehydrogenase activities as indicators of plant stress caused by oxygen deficiency.
- Assays of antioxidant enzyme activities including superoxide dismutase, peroxidase, catalase and glutathione reductase.

- Ultrastructural analyses of cell organelles and aerenchyma development used as morphological markers of hypoxia.

Estimation of the structural alterations occurring in the tested plants together with the changes in the activity of specific marker enzymes and cytosol Ca^{2+} concentration will allow obtaining information about the oxygen deficiency that may appear under microgravity conditions. This will help to search for approaches to overcome this damaging effect that could occur during plant cultivation under microgravity.

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