ISBN: 978-3-95450-168-7 doi:10.18429/JACoW-IPAC2015-TUPWI005

PROTON IRRADIATIONS OF MICRO-TOM RED HAIRY ROOTS TO MIMIC SPACE CONDITIONS

M. Vadrucci, G. Bazzano, L. Picardi, P. Nenzi, C. Ronsivalle, V. Surrenti,
ENEA Radiation Sources Laboratory, Research Center Frascati, Rome, Italy
E. Benvenuto, A. Desiderio, S. Massa, C. Snels, M.E. Villani,
ENEA, Biotechnology Laboratory, Research Center Casaccia, Rome, Italy
F. Ambrosini, Sapienza University of Roma - DIET, Rome, Italy

Abstract

The purpose of the BIOxTREME project, launched by ENEA (Italian National Agency for New Technologies, Energy and Sustainable Economic Development) and funded by ASI (Italian Space Agency), is to formulate new biological drugs having a stimulant activity on the immune system, finalizing the production for a "ready to use" resource in Bioregenerative Life Support Systems (BLSSs) for space missions intended for long-term duration, in deep space, and with multiple crews.
One of the project tasks is to study the effects of physical insults on plants, trying to simulate cosmic environment on the production platforms exposing biological material to ionizing radiation, static magnetic fields and microgravity.

In order to examine the biological effects, to test plant radio-resistance and to build dose-response curves we carried out proton irradiations of anthocyanin rich (red) "hairy roots" derived from the tomato cultivar Micro-Tom with different proton energies for several doses with the TOP-IMPLART accelerator at the ENEA Frascati Research center.

The biological samples were placed in a holder specially made in a movable real-time monitoring chamber calibrated in dose. The fluence-homogeneity measurements over the sample and the calibration of the monitoring system were performed using GafChromic EBT3 films, placed in air in the same position as the biological samples to be irradiated. The present paper describes the experimental set-up and reports the preliminary results.

THE BIOXTREME PROGRAM

Plants can be considered as natural 'bioreactors' for the production of bioactive molecules (e.g. amino acids, vitamins, pigments, antioxidants, complex carbohydrates, proteins, etc.) and biotechnological drugs also including adjuvants or immuno-stimulating molecules.
An important field of interest is the farming of plants in extreme conditions such as those found in the aerospace environment: the prospect of breeding plants in the Space is an essential requirement for long-duration exploratory-class manned missions because they would provide an important physical support as a resource ready for use in Bioregenerative Life Support Systems (BLSS).

The International Space Station and its crew in Space are subjected to galactic cosmic rays (GCR) and solar particle events (SPE). The GCR spectrum is composed primarily of high-energy protons and atomic nuclei, namely about 87% high energy protons, 12% alpha-particles and 1% heavier ions up to iron (HZE) instead SPE consist of low to medium energy protons and alpha-particles. Numerous Space flight and ground-based experiments have been performed to study the biological effects of cosmic radiation on humans in the perspective of manned space missions.

In 2014 ASI has funded the ENEA-project BIOxTREME (BIO-plant factories for the formulation of bioactive molecules endowed with microbicidal, immunostimulating and antioxidant activity, for life in extreme conditions) which aims to formulate new drugs for the astronauts involved in long-term space missions. One of the key features of these drugs is that they have to induce stimulating activity on the immune system through the optimization of the production of natural antioxidants present in plants [1].

The BIOxTREME project is devoted to simulate cosmic conditions exposing plant samples in proton beams, gamma rays, static magnetic fields and microgravity. One of the experimental objectives of the Biotechnology Laboratory at ENEA is the study of alterations in the normal physiology of the living plants in such extreme conditions by profiling biological effects according to "SYSTEM BIOLOGY" technologies, such as proteomics.

In the framework of this program, irradiation of red Micro-Tomato (Micro-Tom) "hairy roots" was carried out with low energy proton beams extracted from the TOP (Oncological Therapy with Protons)-IMPLART (Intensity Modulated Proton Linear Accelerator for RadioTherapy) linear accelerator in operation at ENEA Radiation Sources Laboratory [2].

MICRO-TOM 'RED' "HAIRY ROOTS"

Micro-Tom has been considered the best choice for use in BLSS during human spaceflights. It is a Tomato cultivar (*Solanum lycopersicum*) originally created for ornamental purposes [3] and further widely used as a model species in several studies that defined in detail of the genetic and physiological makeup of this plant. The dwarf phenotype (plant height= 15 cm approx.) is undoubtedly an advantage for growing in small spaces. The miniaturization was obtained by introducing three recessive mutations in the genetic background of tomato

ISBN: 978-3-95450-168-7 doi:10.18429/JACoW-IPAC2015-TUPWI005

[4]. The life cycle of this cultivar is very short and this feature has double advantage of both good productivity while a limited exposure to possible mutations (highly valuable in an environment surrounded by radiations) [5]. Among the significant aspects, there is also the possibility of farming in closed environments, under fluorescent/LED light and in hydroponic conditions (on an inert substrate or directly in water containing salts and nutrients), which make it possible to envisage the cultivation in space facilities already designed as Agrospace Cultivation or Lunar Greenhouse [6].

Generation of "hairy root" Cultures

As a first approach to test harsh conditions we chose not to adopt the whole plant, rather a contained sterile system of organ culture, tailored to this purpose. It is well established that the "hairy root" culture is a great test system for aerospace conditions [7, 8]. Moreover, the use of contained plant systems like "hairy root" cultures for the bioproduction of valuable molecules represents a powerful platform, in that it combines the benefits of cultivating whole-plant (or organ) systems in a controlled environment typical of cell cultures [9].

In close cooperation with University of Amsterdam (Prof. R. Koes and F. Quattrocchio) a plant-expression vector harbouring a Myb-like transcription factor from Petunia in fusion to the reporter gene Green Fluorescent Protein (GFP) was used to constitutively express this transcription factor. In *Petunia,* the cooperation between different transcription factors activates genes involved in the biosynthesis of anthocyanin, responsible for the red pigmentation of petals.

To try to activate the same genetic switch in tomato, hence to build-up a tailor-made anthocyanin-fortified tomato cell system we adopted the well known Agrobacterium-mediated gene transfer. To this aim fully expanded leaves of 5 to 6-week-old Micro-Tom plants, grown at 22°C on a 16 h light/8 h dark cycle, were harvested, surface sterilized, cut into squares, and then exposed to *Agrobacterium rhizogenes* (strain A4RSII) harbouring the foreign gene construction to be transferred for the initiation of "hairy root" tissues.

After infection with *A. rhizogenes*, leaf explants were maintained at 22°C for 48 h in the dark on Murashige and Skoog (MS) medium in 100 mm x 20 mm cell culture Petri dishes (Corning Inc.) and transferred to MS medium containing cefotaxime (Cef, 500 mg/l) and Kanamicyn (Kan, 50 mg/l) (to control bacterial growth and to select transformed plant cells, respectively) solidified with 0.8% agar and incubated at 22°C under a 16 h light/8 h dark cycle for 2 weeks. Actively growing "hairy roots" were individually transferred onto fresh medium and further cultured every two weeks on MS, Kan50 progressively halving Cef concentration at any sub-culture. Production of the red pigmentation was assessed in "hairy root" lines by visual inspection and confirmed for GFP expression by fluorescence microscopy (Zeiss M2 Bio Fluorescence Microscope, Carl Zeiss, Jena, Germany).

The red clonal root line selected for the establishment of technical prerequisites for cell irradiation experiments was grown in polystyrene Petri dishes 85 mm diameter x 13 mm height, 1mm thickness (Phoenix Biomedical), and transferred onto a 15 ml MS Kan50 solid medium (4 mm thickness) one week before the experiment to allow acclimatization into the final container before the experiment.

To avoid variability among biological samples, 3 replicates for each irradiation point were planned as well as a not irradiated control undergoing the same manipulation.

IRRADIATION FACILITY AND PROCEDURE

The experiments were carried out at the TOP-IMPLART facility [10] under realization in the framework of a project led by ENEA in collaboration with Italian Institute of Health (ISS) and Regina Elena National Cancer Institute (IFO-Rome) aimed at realizing a protontherapy centre at IFO. The facility is based on a completely linear proton accelerator consisting of a low frequency (425 MHz) 7 MeV injector (ACCSYS-HITACHI PL7 model, RFQ+DTL) followed by a beam transport line (LEBT) with four electromagnetic quadrupoles, matching the beam to a high frequency (2997.92 MHz) linac booster up to 230 MeV. The segment up to 150 MeV is under construction at the ENEA Research Center in Frascati chosen as test site for its validation before the transfer to IRE-IFO-Rome planned as clinical end user.

At the present time, the part up to 11.6 MeV is fully operational [11, 12] and two beam extraction points are available: the end of a vertical beamline placed after a 90 deg bending magnet located between the two quadrupole doublets and the exit of the first high frequency accelerating module SCDTL-1 (Fig. 1). The irradiations of the μ-TOM 'red' hairy roots were performed in these two points in the operating conditions described below.

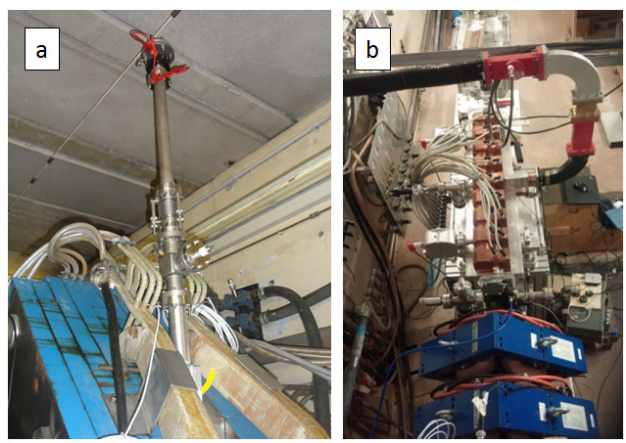

Figure 1: TOP-IMPLART accelerator at ENEA. a) 7MeV protons vertical transport line; b) second pair of quadrupoles and SCDTL-1.

ISBN: 978-3-95450-168-7 doi:10.18429/JACoW-IPAC2015-TUPWI005

First Experiment: 7MeV Protons

The first experiment has been done on the vertical line. The line (70 cm long) is mainly devoted to radiobiology experiments on cells and includes a 2 mm aluminium collimator, a 2 μm gold scatterer spreading uniformly the beam on an area of 13 mm diameter (Fig. 1a) [13]. In the experiment on "hairy roots" the sample holder was horizontally positioned on this vertical terminal: before reaching the sample the beam passes through a 50 μm kapton vacuum/air window, 1.5 cm air and a 60 μm mylar sheet placed on the basis of the Petri dish and invests the μ-TOM red hairy-roots with energy of 5 MeV. The Petri dish is divided into 4 distinct areas in which irradiations were done at 4 different doses: 1.5, 3, 5, 8 Gy reached with $10^5 \div 10^6$ protons/cm^2 and dose rate of 0.2 Gy/s. The dosimetry control was performed with films EBT3 suitably calibrated for the low energy protons [14].

Second Experiment: 11.6 MeV Protons.

The second experiment has been done on the horizontal line positioning the sample holder at the end of SCDTL-1 (Fig. 1b) from which are extracted in air after a 50 μm Kapton window degrading the beam energy from 11.6 MeV to 11.33 MeV. A 30 μm aluminum foil has been inserted to absorb the low energy tail in the proton beam spectrum. At a distance of 34 cm in air the proton beam crosses a lead collimator of 6 mm diameter that kills RX. The Petri dish is placed at 1 cm in air after the collimator on a XY squaring connected to two stepper motors (driven by a Phydget system and remotely controlled via a Labview program specially written) scanning the sample with respect to the beam. The energy of the proton beam on the roots, after 60 μm mylar sheet, is 9.1 MeV. Samples are irradiated in the 1 – 40 Gy dose range with 0.1 Gy/s dose rate. Dosimetry control is carried out by EBT3 films.

EXPERIMENTAL RESULTS

The low energy (5 and 9 MeV), high Linear Energy Transfer (LET), proton beam radiation induces the production of free radicals when interacting with the biological medium. The effect of these radicals is reversible/irreversible impair of fundamental mechanisms of plant cells and could affect morphology, anatomy, biochemistry, and physiology of plants differentially, depending on the irradiation dose.

At the moment, data of our experiments at the early stage deal only with the morphological changes of irradiated roots varying dose and energy of the proton irradiation.

We noticed that the roots that have received an insult from protons of lower energy (5 MeV) do not suffer evident damages: their structures and their hairy extremities resist to proton radiation. Post-exposure observation done in few weeks showed that the effect of the irradiation, compared to the not irradiated control samples, is positive relative to roots growth and expansion: the roots continue to grow in their culture medium and the growth rate is apparently greater than that of the not irradiated control. We also noticed that the expression of anthocyans, which give the typical red colour to the roots (Fig. 2), is greater in the irradiated roots with respect to not irradiated control. A further important consideration relates to the effect of the absorbed dose: in the dose range investigated, we observed that increasing the dose improves performances of the tissues as above described (growth, growth rate and expression of anthocyans).

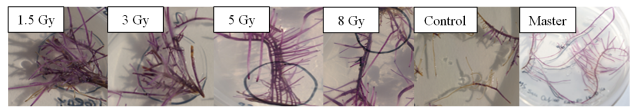

Figure 2: μ-Tom red "hairy roots" irradiated with 5MeV protons at 1.5, 3, 5, 8 Gy, control and master.

Roots receiving the 9 MeV proton irradiation show a similar behaviour (Fig. 3). Samples irradiated with 2.5 Gy have a greater root growth rate, but almost the same density and colour of the control samples. 5 Gy irradiation seems to be the most beneficial one, with the greatest root growth rate and production of anthocyans among the irradiated samples and against the control samples. The 10 Gy sample instead represents the most suffering one, with a poor root growth rate and a pale red colour. Nevertheless it seems that 10 Gy is not a threshold dose, as the 40 Gy sample shows again some benefits with respect to the control samples.

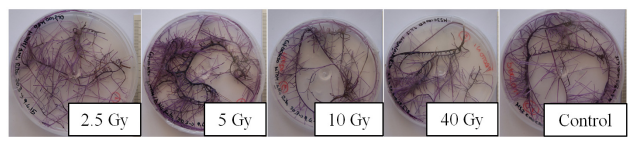

Figure 3: μ-Tom red "hairy roots" irradiated with 5MeV protons at 2.5, 5, 10, 40 Gy, control and master.

In general it is observed from Figures 2 and 3 that the effect of the dose is to increase the roots growth rate (except for the sample irradiated at 10 Gy with 9 MeV protons).

CONCLUSIONS

The results of two pilot experiments, carried out with 5 MeV and 9 MeV protons irradiating μ-Tom red "hairy roots", show that the roots growth rate increases with the energy of protons.

The analysis need to be confirmed by a new experimental campaign which identifies a threshold dose for roots survival and growth effects.

In the matter of energy effect will be essential to make a comparison with experimental results obtained in the framework of BioxTreme project by other irradiation experiments: irradiation with ^{60}Co gamma rays and RX, and also with future more energetic protons (18 MeV protons produced by the under commissioning second SCDTL module of the TOP-IMPLART accelerator).

ISBN: 978-3-95450-168-7 doi:10.18429/JACoW-IPAC2015-TUPWI005

REFERENCES

[1] BioXtreme Project. http://bioxtreme.casaccia.enea.it/

[2] C. Ronsivalle et al., *The TOP IMPLART Project*, Eur. Phys. J Plus (2011) 126, number 7, 68.

[3] Scott JW, Harbaugh BK Micro-Tom. A miniature dwarf tomato. Florida Agricultural Experimental Station 1989; Circular S-370:1-6.

[4] Martí E, Gisbert C, Bishop GJ, Dixon MS, García-Martínez JL Genetic and physiological characterization of tomato cv. Micro-Tom J Exp Bot. 2006; 57(9):2037-47. Epub 2006 May10.

[5] De Micco V, Paradiso R, Aronne G, De Pascale S, Quarto M, Arena C. Leaf anatomy and photochemical behaviour of *Solanum lycopersicum* L. plants from seeds irradiated with low-LET ionising radiation. ScientificWorldJournal 2014: 428141. doi: 10.1155/2014/428141. Epub 2014 Apr 23.

[6] Controlled Environment Agriculture Centre, University of Tucson, Arizona, USA, http://cals.arizona.edu/ceac/

[7] Iversen TH, Odegaard E, Beisvag T, Johnsson A, Rasmussen O. The behaviour of normal and agravitropic transgenic roots of rapeseed (Brassica napus L.) under microgravity conditions. J Biotechnol. 1996 47 (2-3):137-54.

[8] Odegaard E, Nielsen KM, Beisvag T, Evjen K, Johnsson A, Rasmussen O, Iversen TH. Agravitropic behaviour of roots of rapeseed (Brassica napus L.) transformed by Agrobacterium rhizogenes. J Gravit Physiol. 1997 4(3):5-14.

[9] Franconi R, Demurtas O and Massa S 'Plant-derived vaccines and other therapeutics produced in contained systems'. Expert Review of Vaccines (2010) 9 (issue 8): 887-892.

[10] C. Ronsivalle, et al., Proceedings of IPAC2011, San Sebastián, Spain, p. 3580-3582.

[11] L. Picardi, A. Ampollini, G. Bazzano, P. Nenzi, C. Ronsivalle, V. Surrenti, M. Vadrucci, Proceedings of IPAC2014, Dresden, Germany,p. 3247-3249.

[12] L. P. Nenzi, A. Ampollini, G. Bazzano, F. Marracino, L. Picardi, C. Ronsivalle, V. Surrenti, M. Vadrucci., "Status of the TOP-IMPLART Proton LINAC", MOPH066 these Proceedings.

[13] M. Vadrucci, et al., Proceedings of IPAC2014, Dresden, Germany, p. 2156-2158.

[14] M. Vadrucci, et al. Calibration of Gafchromic EBT3 for absorbed dose measurements in low energy proton beam and ^{60}Co γ rays, submitted to Medical Physics Jan 2015.