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Thesis

Application of balloon type bubble bioreactor for micropropagation of *Aloe barbadensis* and *Lilium* hybrid

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ABSTRACT

The objective of the research was to improve the application of balloon type bubble bioreactor for plant micropropagation of Lilium hybrid and Aloe barbadensis. Bioreactor provides optimum growth conditions by regulating chemical or physical parameters to achieve either maximum yield and high quality of the propagules, or to keep the production costs as low as possible by integration of automated facilities and simple low-cost devices. An experiment was performed in order to describe a method for micropropagation of pollenless Asiatic hybrid lilies taking into consideration the genotype, the culture medium and the scale size.

A plant micropropagation protocol for Lilium using an automated balloon type bubble bioreactor (BTBB) was set up. Lilium bulblets were grown in two different culture methods: continuous immersion and temporary immersion in liquid medium (ebb and flood system). Results revealed that morphological traits and biomass accumulation were more efficient when bulblets were cultured in continuous immersion bioreactor. To reduce the risk of contaminations during bioreactor culture, the sterilization of *Lilium* clusters with sodium hypochlorite before setting up the bioreactor and the use of cefotaxime in the liquid medium were of *Lilium* were applied during the in vitro culture. Even it was examined the effect of ozone sterilization in bioreactor culture of *Lilium.* In addition it was examined the dynamic of *Lilium* bulblet growth for the optimum culture period. Results indicated that the use of ozone in bioreactor culture of *Lilium* was an efficient instrument to eliminate the contaminations in the liquid medium without reductions of morphological parameters. Only low signs of oxidative stress were observed in bulblets treated with ozone.

The influence of ozone treatments on *in vitro* propagation of *Aloe barbadensis* in continuous immersion bioreactor was studied. Results provide experimental evidence that the application of continuous immersion bioreactors for the micropropagation of *A. barbadensis* is a viable and efficient model for automation and large-scale production of quality shoots.

1 GENERAL PART

1.1 PLANT TISSUE CULTURE

A large number of commercially important plants including important crops such as vegetables, flowers, ornamentals, fruit trees, woody and medicinal plants are vegetatively propagated by tissue culture (Altman and Loberant, 2000). This technology has now been commercialized globally and has contributed significantly towards the enhanced production of high quality planting material.

Plant tissue culture (PTC) broadly refers to cultivation of plant cells, tissues, organs, and plantlets on artificial medium under aseptic and controlled environmental conditions. PTC is the art of growing experimental plants, selecting a suitable plant organ or tissue to initiate cultures, cleaning, sterilization and trimming it to a suitable size, and planting it on a culture medium in right orientation while maintaining complete asepsis. It also requires an experienced and vigilant eye to select healthy and normal tissues for subculture (Bhojwani and Dantu, 2013). Tissue culture methods have also been employed to study the basic aspects of plant growth, metabolism, differentiation and morphogenesis and provide ideal opportunity to manipulate these processes (Gupta and Ibaraki, 2006). PTC involves a scientific approach to systematically optimize physical (nature of the substrate, pH, light, temperature and humidity), chemical (composition of the culture medium, particularly nutrients and growth regulators), biological (source, physiological status and size of the explant), and environmental (gaseous environment inside the culture vial) parameters to achieve the desired growth rate, cellular metabolism, and differentiation (Bhojwani and Dantu, 2013). PTC is also the best technique to exploit the cellular totipotency of plant cells for numerous practical applications, and offers technologies for crop improvement (haploid and triploid production, in vitro fertilization, hybrid embryo rescue, variant selection), clonal propagation, virus elimination, germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization) (Bhojwani and Dantu, 2013). In the years to come, the application of plant tissue culture for various biotechnological purposes will increasingly depend on the adoption of engineering principles and better understanding of their interacting factors with biological system (Gupta and Ibaraki, 2006).

Tissue culture is carried out in most of countries in the world, and the number of plants propagated was 600 millions for one year over the world which is the best available estimates as cited in Altman and Loberant (2000). The culture technique generally used for commercial tissue culture propagation is the agar culture which requires large number of small culture vessels and labour, periodic transfers of plant material to fresh media, after exhaustion of the nutrients in the medium and also because of continuous tissue growth and proliferation, which is rapidly limited by the size of the culture container and results in the requirement of many laminar air flow clean benches, large autoclave(s), large culture spaces equipped with illuminated shelves, electric energy, etc. This is the major cause for both limited propagation efficiency and high production costs (Maene and Debergh, 1985; Takayama and Akita, 2006; Adelberg and Fari, 2010). As well the major disadvantage of the conventional micropropagation techniques is the difficulty in controlling the chemical and/or physical properties in culture vessels (Paek et al., 2001; Paek et al., 2005).

In order to overcome these problems, it is important to develop new propagation strategies to overcome the limitations of conventional micropropagation techniques. Many attempts for establishing large-scale production of propagules with simple production facilities and techniques have been made including robotics, photoautotrophic cultures, bioreactor techniques, etc. (Takayama,1991).

Liquid culture systems

In recent years, liquid culture systems based on shoot cultures or somatic embryos have become of increasing interest to commercial micropropagators for some stages of the plant propagation cycle. Various vessels have been investigated for liquid cultures, from simple devices supplying an arbitrary amount of oxygen, to complex computer-controlled bioreactors that have been especially designed for plant cell multiplication and regeneration (Hvoslef-Eide and Preil, 2005).

1.2 BIOREACTORS FOR PLANT PROPAGATION

The term “bioreactor” is not exactly defined is generally describe as specialized tissue culture vessels that provide readily accessible nutrients and water throughout the duration of a culture cycle (Adelberg and Fari, 2010). Bioreactors are widely used for plant biomass production, and in the cultivation of organisms such as microbes, animal

or plant cells to produce metabolites or cells (Coombs, 1986; Takayama, 2000; Lian et al., 2003a,b; Hahn and Paek, 2005; Ziv, 2005; Dewir et al., 2006; Takayama and Akita 2006; Cui et al., 2010a,b). Bioreactors are normally connected to units controlling temperature, pH, aeration, stirring and various other devices. However, in some cases *in vitro* culture vessels that deviate from erlenmeyer flasks, petri dishes or culture boxes are called "bioreactors", too, when innovations and improvements of vessel design were introduced. (Preil, 2005).

1.2.1 ADVANTAGES OF BIOREACTOR CULTURE SYSTEMS

The aim of bioreactor application is to provide optimum growth conditions by regulating chemical or physical parameters to achieve either maximum yield and high quality of the propagules, or to keep the production costs as low as possible by integration of automated facilities and simple low-cost devices (Preil, 2005). Since 1980 several publications have demonstrated the advantages of bioreactors and discussed diverse designs or application strategies (Ammirato and Styer, 1985; Styer, 1985; Preil 1991; Takayama, 1991; Denchev et al., 1992; Takayama and Akita, 1994, 1998; 2006, Heyerdahl et al., 1995; Son et al., 1999; Ziv, 2000; Peak et al., 2001; Paek et al., 2005; Ziv, 2005).

The system culture offers many advantages, including: Better control of the culture conditions, bioreactor culture systems provide a maximal opportunity for monitoring and control over microenvironmental conditions (agitation, aeration, temperature, dissolved oxygen, pH, etc.) (Paek et, 2001; Paek et al., 2005; Ziv, 2005); large numbers of plantlets are easily produced, and scaling up and automation of micropropagation are possible (Aitken-Christie, 1991; Paek et al., 2005; Berthouly and Etienne, 2005; Takayama and Akita, 2006; Adelberg and Fari, 2010); since handling of cultures such as inoculation or harvest is easy, ability to work with larger vessels, reducing the number of culture vessels, and the area of culture space results in the reduction of costs (Takayama and Akita, 2005; Takayama and Akita, 2006; Adelberg and Fari, 2010); cultures are always in contact with the medium facilitating uptake of nutrients, optimal supply of growth regulators and growth rate (Takayama and Akita, 1994; Takayama and Akita 2005; Ziv, 2005); forced aeration (oxygen supply) is performed, which improves the growth rate and final biomass achieved (Takayama and Akita, 1994; Takayama and

Akita, 2005); bioreactor systems provide much more uniform culturing conditions, cultures are moving in the bioreactor, which results in the disappearance of apical dominance and in the development of numerous shoot buds into plantlets (Takayama and Akita, 1994; Berthouly and Etienne, 2005; Takayama and Akita, 2005).

Compared to agar-based systems, liquid systems are more adaptable to automation and are, therefore, suitable for the reduction of labour and costs, liquid culture systems provide much more uniform culturing conditions, the media can easily be renewed without changing the container, and container cleaning after a culture period is much easier. In addition, with liquid culture media, much larger containers can be used and more of the container volume can be used, whereas agar media necessitate flat culturing (Berthouly and Etienne, 2005; Preil, 2005).

A very practical advantage to liquid culture in large vessels is “cut and dump” vessel filling at the transfer hood (Adelberg and Fari, 2010). Manual labour in hood transfer is about 45% of hood time was spent orienting, spacing and plating the cuts in the new vessel of gelled medium (Alper et al., 1994; Adelberg and Fari, 2010). In liquid, transfer times can be reduced since explants are no longer positioned, plantlets are not oriented when placed in the vessel; dozens of cut plantlets may be introduced is one motion, and passive spacing occurs when the liquid is agitated. In this “cut and dump process” vessel with larger numbers of plants are more efficiently processed at the transfer station than small jars (Berthouly and Etienne, 2005; Adelberg and Fari, 2010)

The greatest problem with gelling agents is that nutrients dissolved in the liquid phase are not completely available as water is bound to the gel by a matric force. Liquid medium has greater water availability than agar, and the matric component of water potential may underestimate agar’s effect on water potential (George et al., 2008; Adelberg and Fari, 2010). In larger vessels of agar, the higher sucrose concentrations created more negative water potentials and decreased water availability, in a shaken liquid culture, the entire volume is at equilibrium and there is no distinction between source and adjacent medium. Interfacial transfer from the medium to the plant is quantified by a second resentence term. The resistance of sucrose transfer from agar is 300 X greater than that of sucrose transfer from a shaken liquid to the plant, per unit surface area (Adelberg and Fari, 2010).

Moreover, plant tissues from numerous species have performed better when cultured in liquid medium rather than on an agar medium. For instance, a larger number of shoots were produced in *Prunus persica* L. Batsch (Hammerschlag, 1982). More somatic embryos were produced in soft-red winter wheat (Jones and Petolino, 1988) and *Gossypium hirsutum* (Gawel and Robacker, 1990), (Berthouly and Etienne, 2005).

1.2.2 DISADVANTAGES OF BIOREACTOR CULTURE SYSTEMS

The use of liquid cultures in bioreactor for plant propagation imposes several problems such as: Leakage of endogenous growth factors, the need for an initial high concentration of the inoculum, hyperhydricity and malformation of shoots, plantlet size variation, foam development, shearing and oxidative stress (Takayama and Akita, 1998; Ziv, 2000); large initial investment for equipment includes substantial costs in repair, replacement parts and upkeep of bioreactor facilities (Paek et al., 2001); the existence of recalcitrant species for bioreactor application and such species are difficult to be cultured in liquid medium even if they are possible to be propagated on agar medium (Takayama and Akita, 2006); the high risk of contamination of the system, the microbial contamination is frequently observed in laboratory and commercial tissue cultures, and when contamination is introduced to a large batch of propagules, is likely to lead to total loss of the culture and the cost and lost time can be devastating (Leathers et al., 1995; Preil 2005; Takayama and Akita, 2006, Luna et al., 2009; Cardona Suarez et al., 2012). In addition, liquid systems often result in bottlenecks or limiting factors greater than those encountered using gelled media. All users of plant cell, tissue or organ cultures in liquid media face similar problems, irrespective of species with which they work; namely, problems related to the initiation and development of cultures and their liquid environment; how to supply sufficient oxygen; how to maintain asepsis; and how to maintain genetic quality in spite of somaclonal, epigenetic and physiological variations. Some of these issues are more prominent in liquid culture systems, compared with those on gelled media; some may be less prominent (Hvoslef-Eide and Preil, 2005).

1.2.3 TYPES OF BIOREACTORS FOR PLANT PROPAGATION

Various types of bioreactors are used for this purpose which are classified by agitation methods and vessel construction into; mechanically agitated bioreactors (aeration-

agitation bioreactors, rotating drum bioreactors, spin filter bioreactors), pneumatically agitated bioreactors (unstirred bubble bioreactor, bubble column bioreactor, air-lift bioreactor), and non-agitated bioreactors (gaseous phase bioreactor, oxygen permeable membrane aerator bioreactor, overlay aeration bioreactor) (Takayama and Akita, 1994). Mechanically agitated bioreactors, the most standardized bioreactor system in industrial processes, are applicable to plant propagation. However, pneumatically driven bioreactors such as bubble column, unstirred bubble and airlift bioreactors are found to be suitable as plant bioreactors because it compensate the specific problem of mechanically agitated bioreactors such as severe shear generation. The most frequently used bioreactors having the characteristics suitable for plant organs, especially for shoot cultures are unstirred bubble bioreactors, bubble column bioreactors and airlift bioreactors (Takayama and Akita, 2006).

Airlift and bubble column-type bioreactors

The merits of airlift devices are (a) low shear stress, (b) simplicity of their design and construction, (c) availability for long-term culture with rare contamination by eliminating the risks caused by stirrer shafts and seals, (d) comparatively low amount of energy consumption. The major difference between the airlift and the bubble column reactors is provision of a circulation system and hydrodynamic behavior. In the airlift bioreactor, liquid circulation and mixing are determined by the gas flow rate, therefore, liquid circulation velocity controlled by an air flow meter may be generated without any other external circulation mechanism compared to those of a bubble column bioreactor. Although the lifting mechanism is almost similar between the two systems, the bubble column bioreactor is more suitable for large-volume cell cultivation than the airlift bioreactor. Many modifications in their configuration may improve the efficiency of bioreactors. This may include (a) several designs of the horizontal connections at the place where the medium rises and falls by air circulation, (b) head-space region for gas-liquid separation, (c) rectangular and square cross-sections, (d) gas sparger, (e) the angle between sparger and main vessel (Peak et al., 2001)

The disadvantages of airlift and bubble column-type bioreactors are foaming induced by large volumes of air, a tendency of cells to be thrown out of the solution by bubbles or air, and wall growth of cells in the foam in the headspace. The quantity of cells contained within this region represents a significant quantity of total biomass. The problem of foaming appears in nearly all plant cell cultures, but may be worse in some

cell lines than in others. To exclude foaming, antifoam agents such as silicone-based products can be used, but it is strongly recommended to check their effects on culture growth; cell viability and metabolite production (Leathers et al., 1995; Lee, 1997).

The phenomenon of foaming and cell wall growth in airlift or bubble column-type bioreactor cultures originates from the fact that the diameters of the vessel and the top of the section are the same. We solved this problem by designing improved models of airlift and/or column-type bioreactors that have a larger top section diameter and/or balloon-type bioreactors. Another problem of airlift bioreactor culture is evaporation of culture medium. To overcome this problem, a sterile water column should be introduced between the sterilized membrane filter and the glass sparger. By the incorporation of a water column, evaporation of culture medium was reduced by 90%, and as a result, the cultivation period could be extended (Lee, 1997).

Balloon-type bubble bioreactor (BTBB)

BTBB was designed as follow: in the bottom of the reactor a `Y'- or a `T'-shaped tube was attached as inoculum and harvest ports. By using a concentric tube for cell lifting at the riser part of the vessel bottom, foaming was drastically reduced, resulting in the reduction of cell wall growth. Also, incorporation of a short cylindrical hole on the top section of the BTBB allowed easy culture and harvest of culture tissues such as adventitious or hairy roots. In addition, the stopper on the hole provided space to install extra ports for the measurement of pH and dissolved oxygen (DO). Introduction of a gas-recycling system allowed the examination of various gases in the bioreactor (Peak et al., 2001). With extensive research, the BTBB was found to be a reliable bioreactor system for continuous cell culture and for promotion of cell growth (Seon et al., 1998). Son et al. (1999a, b) designed and constructed pilot-scale BTBBs of 300 and 500 liters for the production of biomass or valuable secondary metabolites

Stirred tank bioreactor (STR)

One of the most popular industrial-scale bioreactor systems is the mechanically agitated STR. It has advantages in terms of effectiveness in mixing and breaking-up the air bubbles that prevent large cell aggregates and enhance oxygenation (Hooker and Lee 1990). On the other hand, it has some disadvantages such as high shear force, complicated configuration, great exposure to contaminants, difficulty in optimizing

variable conditions, and high energy consumption. Unlike pneumatic bioreactors, the STR has a separate mechanism for mechanical agitation using an impeller and aeration. Cultured plant cells and propagules that are sensitive to hydrodynamic shear stress require low shear-associated impellers that provide good mixing at relatively low agitation rates (Leathers et al., 1995).

Typical stirrers are propellers and pinched-blade turbines that cause axial fluid motion. Flat blade turbines and impellers produce radial fluid flow. Paddle agitators cause tangential flow as well as radial flow (Schiigerl and Sittig, 1987). The effects of the blade number, internal position of blades, and the ratio of diameter of the stirrer and vessel need to be determined to ensure optimum conditions, especially for shear-sensitive cells (Lee, 1997).

To maintain sufficiently high oxygen transfer rates, sparging systems, sintered glass and porous steel devices with pore diameters of approximately 10 mm are used. These devices are capable of generating microscopic bubbles with a large interfacial area for gaseous diffusion. For aeration purposes the non-stirred tank bioreactor adapts a standard airlifting sparging system supplemented with a bubble-free aeration mechanism consisting of coils of porous silicone tubing wound around the outside of the draught tube which are fed with a pure oxygen supply (Preil et al., 1988). The oxygen concentration in the medium can be regulated by means of pulse-modulated dosage of oxygen through an open-ended tubing or through a closed circulation system agitated by a peristatic pump. The bubble-free aeration method is very efficient in providing oxygen during suspension cultures and in eliminating negative effects of air bubbling (Peak et al., 2001).

Notwithstanding, the STR presents several limitations such as high power consumption, high shear forces and problems with sealing and stability of rotating shafts in tall bioreactors. Air-lift bioreactors combine high loading of ‘solid’ particles and good mass transfer, which are inherent for three-phase fluidized beds. Air bubbles, using internal or external recirculation loops, generate efficient mixing in the liquid phase. The main advantages of air-lift bioreactors are low shear forces, low energy requirements, and simple design. Rotary drum reactors have significantly higher surface area to volume ratios than other reactor types (Paek et al., 2005).

Ebb and flood bioreactor – (periodic immersion system)

The principal equipments in an ebb and flood bioreactor are the same as those in airlift or bubble column-type bioreactors. However, a fixed or floating raft support system inside the culture vessel is required to support the plant tissues. In the modern ebb and flood system, medium is pumped from a storage tank into culture vessels. A series of channels helps to supply nutrient solution evenly to the plant materials, resulting in uniform growth. The medium remains in the vessel for a few minutes, after which it drains back to the storage tank for reuse The drainage process is controlled by a solenoid valve at intervals of between 4 and 8 h, depending on plant species and explant type (Peak et al., 2001; Paek et al.,2005).

In an ebb and flood bioreactor system, each step of plantlet production can be carried out in the same culture vessel simply by changing the culture medium. The nutrient outflow is controlled with a metering pump and the culture medium level is regulated by a level controller, which facilitates manipulation of cell and tissue growth by controlling the ratio of outflow to nutrient medium fed. In addition, short-term exposures to plant growth regulators, high concentrations of cytokinin and/or auxin, for the induction of embryogenesis and organogenesis, can be performed more precisely. This system completely eliminates the subculture stage in vitro, which is the most labor-intensive, and therefore the most costly stage of micropropagation (Peak et al., 2001; Paek et al., 2005).

Temporary immersion systems **TIS**

A periodic immersion technique in which the plant tissue spends periods immersed in the liquid medium alternating with periods in the air was first described by Steward et al. (1952) and modified by Steward and Shantz (1956). In the so-called "Steward apparatus" or "Auxophyton", tubes are fastened with their long axes parallel to the radius of discs which are rotated at 1 rpm by a shaft held at an angle of 10-12°. The liquid medium runs from one end of the tube to the other leaving the tissue in the gas phase and *vice versa*. By similar means, Harris and Mason (1983) achieved alternate exposure and submergence of explants when tilting a flat-bottomed vessel in one direction to expose the tissue to air and in the opposite direction to submerge them in the medium. The first automated device operating according to the principle of ebb and flood was designed by Tisserat and Vandercook (1985). This soon initiated discussions on growing *in vitro* cultures which are temporarily immersed. A breakthrough was achieved when Alvard et al. (1993) introduced the RITA® vessel and Teisson and

Alvard (1995) stressed temporary immersion as a new concept of plant *in vitro* cultivation using liquid media. These ideas fertilized worldwide activities in the application and testing of temporary immersion systems (TIS), resulting in variations in vessel design, equipment and treatments such as immersion time and frequency, depending on the requirements of different plant species and cultivars (Preil, 2005).

An overview on TIS was given by Etienne and Berthouly (2002) and Berthouly and Etienne (2005). One can only speculate why this culture technique was adopted so late as a simple, low-cost and effective propagation method, which is applicable to almost all commercially interesting species The principles of the RITA® system and of the twin-flask system of Escalona et al. (1999) represent the most convincing technical solutions combining low-cost devices and easy handling of the cultures. The practical advantages of TIS became evident when culturing e.g. somatic embryos of *Musa* spp. (Escalant et al., 1994), *Hevea brasiliensis* (Etienne et al., 1997b), *Citrus* (Cabasson et al., 1997) and *Coffea arabica* (Etienne et al., 1997a; Etienne-Barry et al., 1999) *Psidium guajava* (Gómez et al., 2005) as well as shoots of sugarcane (Lorenzo et al., 1998), pineapple (Escalona et al., 1998, 1999), *Phalaenopsis* (Hempfling and Preil, 2005), olive (Grigoriadou et al., 2005) and *Charybdis* sp (Wawrosch et al., 2005) or potato microtubers (Akita and Takayama, 1994b; Jimenez et al., 1999).

The physiologically most important advantage of TIS is the efficient gaseous exchange between plant tissue and gas phase inside the vessel. Multiple daily air replacement by pneumatic transfer of the medium ventilates accumulated gasses like ethylene or CO2. Additionally, uptake of nutrients and hormones over the whole explant surface ensures maximum growth. There is no doubt that TIS will play an outstanding role in future mass propagation of plants *in vitro* (Preil, 2005).

The systems differ with respect to container size, type of culture support, existence of computerized immersion control or a simple timer, use of either a peristaltic pump, or an air pump, or mechanical motion of the container to displace the liquid. Other differences between the temporary immersion systems include recycling or not of the medium, and separation or incorporation of the medium tank with the culture container. Characteristics common to these systems include containers that are larger than conventional culture vessels, transparent and autoclavable. These systems are easier to use than conventional bioreactors, and longer subculture periods are possible with most

of them. Temporary immersion culture systems provide programmable partial or total contact between the explant and the liquid medium The systems that have been designed can be divided into the following four categories (Berthouly and Etienne, 2005).

Systems with tilting or rocker machines

Two machines were described by Harris and Mason (1983). The tilting machine inclines Erlenmeyers flasks 30 degrees in opposite directions; it has a capacity of 400 50-ml Erlenmeyer flasks or 320 125-ml flasks. The Rocker machine rolls 70 910-ml wide-mouth jars lying on their sides, or tilts 120 455-ml wide-mouth jars standing upright 30-40 degrees every 30 sec. These machines do not include replenishment of the liquid culture medium (Berthouly and Etienne, 2005).

Systems with complete immersion and a liquid medium renewal mechanism

Tisserat and Vandercook (1985) developed a large elevated culture chamber that was periodically drained and then refilled with fresh medium in a sterile environment. The automated plant culture system consists of silicone tubing, 2 impeller pumps, 2 glass medium reservoir bottles, a 3-way stainless steel valve, a plant culture chamber, and an interface module containing relay boards. This system provides a longterm method for in vitro plant culture (Berthouly and Etienne, 2005).

Systems with partial immersion and a liquid medium renewal mechanism

Explant is always positioned on a culture support agar medium, propylene screen, or cellulose plugs. Liquid culture medium is frequently supplied, then withdrawn into a drain-off recipient vessel, so as to imbibe the support, stabilize the composition of the culture medium and extend the duration of subcultures, whilst avoiding or postponing the need to change the medium. Only the base of the plant material is partially immersed. Two models have been published:

– Aitken-Christie and Jones (1987) and Aitken-Christie and Davies (1988) proposed a semi-automatic process in large polycarbonate containers measuring 250 x 390 x 120mm. In their system, *Pinus* shoots were grown on an agar medium, with automatic addition and withdrawal of liquid medium by peristaltic pumps on a periodic basis. The liquid from the fresh medium recipient vessel came into contact with the explants for 4 to 6 hours, using a vacuum suction system, then went to the drain-off recipient vessel.

– Simonton et al. (1991): their system featured a computer-controlled pumping apparatus that intermittently supplied liquid medium to plants cultured in 7-litre vessels. Plant material rested on a perforated polypropylene screen that was attached to the inside of the vessel. Control capabilities included medium introduction and depth regulation within four individual culture vessels, medium cycling on an assigned schedule, schedule adjustment during a culture period, and medium replacement (Etienne and Berthouly 2002; Berthouly and Etienne 2005).

Systems with complete immersion by pneumatic driven

After the first publication by Alvard et al. (1993) different systems were described These include the most recent temporary immersion systems. They are simple and easy to use. They enable contact between all parts of the explant and the liquid medium, along with complete renewal of the culture atmosphere by forced ventilation, which drives the liquid towards the plant material. The plant material can be placed in the container in bulk, removing the need to position the plant material on a support. Such systems include pneumatic transfer of the medium from a reservoir tank to the container holding the plants. To avoid excess tubing, these two compartments are preferably part of the same vessel. Pressure is applied through a solenoid valve by a compressor connected to a programmable plug. This application determines the time and duration of floodings. As these systems do not include a fresh medium tank, the culture medium has to be changed after 4 to 6 weeks. However, replacement is rapid and there is no need to transfer the plant material. Two variants of this system have been developed and are currently on the market: the Recipient for Automated Temporary Immersion system (RITA ®) and the twin flasks system (BIT®).

The RITA® system (Teisson and Alvard 1995). The 1-litre vessel comprises two compartments, an upper one with the plant material and a lower one with the medium. The pressure applied in the lower compartment pushes the medium into the upper one. Plants are immersed as long as pressure is applied. During the immersion period, air is bubbled through the medium, gently stirring the plants and renewing the headspace atmosphere inside the culture vessel, with the pressure escaping through outlets on the top of the apparatus.

The twin flasks system (BIT®) (Escalona et al., 1998). The easiest way to carry out pneumatically driven temporary immersion is to connect two glass or plastic flasks - from 250 ml to 10 liters - by tubing, and apply alternative pressure to push the medium into the respective recipient vessels. The RITA® vessel can easily be adapted to this configuration. In 1994, Akita and Takayama proposed a similar system known as the 'system for semi-continuous medium surface level control culture', adapted to potato tuberization. That system incorporates a forced aeration in the culture vessel, which is not found in the BIT® System (Etienne and Berthouly 2002; Berthouly and Etienne 2005).

1.2.4 PHYSICAL AND CHEMICAL FACTORS AFFECTING CELL GROWTH

Monitoring of the physico-chemical environment factors is necessary to obtain healthy plantlets, which eventually results in successful acclimatization in the greenhouse conditions. Bioreactors should be equipped with various probes for measurement and control of temperature, agitator speed, medium pH, dissolved oxygen, carbon dioxide and redox potential (Peak et al., 2001; Peak et al., 2005)

pH of the medium

The measurement of pH is necessary in suspension cultures, since changes in pH indicate alterations of internal physical and chemical factors, Changes in pH during culture have been reported by several authors (Dussert et al., 1995; Hilton and Wilson, 1995; Yu et al., 2000; Lian et al., 2002; Shin et al., 2003; Chakrabarty et al., 2007; Jeon et al., 2009; Cui et al., 2010; Yang et al., 2010). These changes appeared to be related to the balance between ammonium in the medium. The high decrease in pH in the culture may be explained by the ammonium uptake, NH_4^+ was rapidly depleted leading to higher acidification of the medium (Chakrabarty et al., 2007). This rapid decrease in pH of the culture media may be due to the efflux of protons during the absorption of NH_4^+ (Dantas et al., 2001; Lian et al., 2002; Chakrabarty et al., 2007). However, pH can be altered not just by the ammonium-nitrate balance but by various other factors. For example, when the aerial oxygen concentration is regulated periodically between 10 and 80%, pH changes by 0.2 pH units depending on the volume of the medium and the size of the culture vessel (Lee, 1997). Precise recording of pH changes during bioreactor

culture will improve the repeatability of complex biological processes (Paek et al., 2001; Paek et al., 2005)

Dissolved oxygen (DO)

In the bioreactor DO concentration can be measured by a sterilizable electrode and it can be controlled by regulation of agitator speed or air flow controller. DO concentration of liquid medium indicates the amount of oxygen available for cell metabolism. Since oxygen is not completely soluble in water, its content is rapidly exhausted when the supply is interrupted, especially at high cell density. Generally, microbial cultures have higher oxygen demands compared to either animal or plant cell cultures. Oxygen transfer coefficient (kLa) during batch cell culture reveals that the value of kLa increases with increasing the number of rotations of agitator and the maximum kLa occurred in the middle of the culture time (Paek et al., 2001). There was a tendency to a low kLa in the early stage of culture at high inorganic nutrient concentration in the medium (Azechi et al., 1985).

In bioreactors one of the main functions is to promote the mass transfer of oxygen from the gaseous to the liquid phase. Since oxygen is only sparingly soluble in water (0.25 mmol ^{1} at 25 ^{o}C. 1 atm, 21% (v/v) O_2 in the air), it is necessary to drive the diffusion of oxygen into the aqueous phase to meet the demand of actively growing tissues or cells (Leathers et al., 1995). This is accomplished by modifying operational parameters such as aeration rate, agitation speed, impeller design, gas mixing and bioreactor configuration. Culture mixing is also important because dissolved oxygen (DO) must be transported rapidly to the culture tissues or cells. In general, it is essential that the dissolved oxygen concentration remains above the critical DO_2 level at all the times for optimal cell growth (Leathers et al., 1995; Sajc et al., 2000). The critical dissolved oxygen concentration, DO_{2crit}, can be described as the dissolved oxygen concentration above which no further increase in specific oxygen uptake rate can be measured. At DO_2 levels below the DO_{2crit}, cells have reduced energy (ATP) levels, which may have direct effects on cellular metabolism and morphology (Leathers et al., 1995). Practically, this is important because the DO_{2crit} value is used for designing appropriate bioreactor operating systems to ensure that an oxygen-limiting condition does not suppress the metabolic activity of the culture. Therefore, supplying adequate amounts of oxygen (above the DO_{2crit}) is a major concern in bioreactor scale-up (Paek et al., 2005).

1.2.4.3. Carbohydrate supply and utilization

Sucrose, and to a lesser extent glucose, fructose, or sorbitol, are the most commonly used carbohydrates in vitro. In general, sucrose is removed rather rapidly from the medium and after 10– 15 days the sucrose can be completely depleted or reduced to 5–10 g l-1 from an initial concentration of 30 g l-1 in both agar-gelled and liquid cultures (Ziv, 2005). At the same time glucose and fructose that appear in the medium due to sucrose hydrolysis increases in the presence of invertase in the culture medium, and can reach concentrations of 5–10 g l-1. For optimal bulblet growth of *Lilium* in bioreactor culture high sucrose levels are necessary (Lian et al., 2002, Lian et al., 2003a), bulblets of *Lilium* grew faster when the medium was exchanged frequently with new medium in a BTBB and sucrose supplied was rapidly hydrolysed into glucose and fructose. However, Cui et al., (2010) found in root suspension cultures of *Hypericum perforatum* L that higher sucrose concentrations (5, 7, and 9%) inhibited biomass accumulation due to the relatively higher osmotic pressure, and increased sucrose concentration resulted in osmotic stress and, in turn, induces the accumulation of secondary metabolites. The biomass of Boston fern meristematic clusters in a bubble-column bioreactor was increased with the increase in sucrose concentrations from 7.5 to 30 g l-1, while higher concentrations caused a decrease in cluster growth. Elevated sucrose concentrations in the medium caused a decrease in the clusters size and leaf chlorophyll content (Ziv and Hadar, 1991). Gladiolus clusters cultured in the presence of growth retardants had a higher concentration of starch – 845 as compared to 585 mg g^{-1} DW in the control (Ziv, 1992). Potato microtubers grew at a faster rate in a rotating bioreactor when the medium was replaced frequently and the number of tubers >1 g increased 4-fold when 8% (w/v) sucrose was used (Yu et al., 2000).

Inoculum density

In order to produce a large number of plantlets in a bioreactor, large numbers of propagules developing new shoots are prepared as inocula. The appropriate propagules include a) multiple shoot buds, b) regenerative tissues such as protocorm-like bodies, embryogenic or meristematic tissues, c) somatic embryos, or d) stems or shoots with a number of axillary buds. Here multiple shoot buds and stems or shoots were used as inocula because of their genetic stability Small pieces of tissue with multiple shoot buds that have been propagated in the test tubes were the most preferable inoculum for the bioreactor (Takayama and Akita, 2005). There is a minimum size of explant or quantity of separated cells per unit culture volume for successful culture initiation. Large

explants generally survive better and grow more rapidly in comparison to relatively small explants at the initial stage of culture. The effect of inoculum density on biomass increase varies with the types of culture, plant species and culture period (Paek et al., 2001). High inoculum density of 20±30% (v/v) is required to assure minimal lag phase times and a significant growth of plant cells (Son et al., 1999a). In *Hypericum perforatum* an inoculum density of 6g/l fresh weight was the optimum size for the shake flask culture growth and secondary metabolites (Cui et al., 2010) and an inoculation density of 20 g (FW) was optimal for production of protocorm like bodies in a 3l working volume of 5 l bioreactor (Yang et al., 2010)

Mixing

Mixing is the other key parameter, which is necessary to distribute equally cells or tissues, and nutrients throughout the liquid phase (Leathers et al., 1995; Sajc et al., 2000; Honda et al., 2001). Mixing is normally carried out by sparging, mechanical agitation or a combination of these two, but the magnitude of hydrodynamic forces associated with mixing should be small enough not to cause cell or tissue damage, but sufficient to stimulate selected cell functions. However, there has been little quantitative work on the effect of hydrodynamic forces on plant tissue engineering (Paek et al., 2005).

Mineral nutrients

Nutrient availability is a major chemical factor involved in scaling up. For large-scale culture in a bioreactor several aspects play an important role. Periodic measurement of the individual nutrients at different times provides information regarding nutrient uptake, biomass and metabolite production in bioreactors. Analysis of the dynamics of various nutrient compounds during *Lilium* bulblet growth in BTBB revealed that ammonium, nitrate and phosphate became exhausted from the medium. After 16 weeks of culture considerable amounts of K^+, Mg2+, Ca^{2+}, Na^+ and Cl^- were still present in the medium, but the limiting growth factor was sugar, rather than the main nutrient (Lian et al., 2002). Similar investigations were carried out during bioreactor culture with Begonia (Tormala et al., 1987), rice suspension culture (Schmitz and Lorz, 1990), carrot somatic embryo (Archambault et al., 1995), potato microtuber (Yu et al., 2000); ginseng adventitious roots (Yu et al., 2001a, b), hairy root of *Beta vulgaris* (Shin et al., 2003) and apple growth (Chakrabarty et al., 2007).

The gaseous atmosphere

Plants cultured aerobically require oxygen for growth. In small scale semi-solid cultures, culture vessels such as flasks or bottles are plugged using gas diffusive materials. Molecular diffusion through plugs allows oxygen to penetrate into culture flasks or bottles, and stimulate the cultures to grow. On the contrary, in case of cultures submerged in liquid medium such as shake or bioreactor culture, natural diffusion of oxygen is limited and plant growth is strictly inhibited without shaking or forced aeration (Takayama and Akita, 2006). Gas composition in the culture vessel is influenced by the volume of the vessel, the volume of the medium and the ventilation. In bioreactors, control of the gaseous phase depends on the gas flow and can be easily manipulated to provide the required concentrations of O_2, CO_2 and C_2H_4. In airlift or bubble-column bioreactors, the air supplied is used for both mixing and aeration. The importance of aeration and the gaseous phase were shown in potatoes cultured in airlift bioreactors (Ziv, 2005). Induction of tubers was inhibited under continuously submerged conditions. Microtubers developed only after the shoots elongated and reached the gaseous phase. A two-phase culture, substituting the growth medium with a tuber-induction (9% (w/v) sucrose) medium, enhanced tuber formation from shoots which developed above the medium and were exposed to the gaseous phase (Akita and Takayama, 1994b).

Oxygen concentrations in liquid cultures depend on the presence of O_2 in the gas phase above and in the air bubbles inside the medium, as well as in the dissolved O_2 in the medium. Air is sparged through a sparger located at the base of the bioreactor. Oxygen requirements may vary from one species to another, and concentration of O_2 in liquid cultures in bioreactors can be regulated by agitation or stirring and through aeration, gas flow and air bubble size. Increasing O_2 concentrations, in the sparging air, from 21 to 80% (v/v), in bioreactor cultures of Boston fern clusters, enhanced growth values (final FW - initial FW)/(initial FW) from 0.61 to 0.92 (Ziv and Hadar, 1991). Reducing O_2 concentrations to 10% (v/v) affected cell differentiation in bioreactor cultures of carrot embryogenic tissue. Under these conditions embryo production was severely inhibited (Jay et al., 1992).

Shearing and oxidative stress

In many plants cultivated in bioreactors, the continuous aeration, mixing and circulation cause shearing damage and cell wall breakdown. The cell debris adheres to the vessel

and causes foaming that prevents adequate liquid circulation and oxygen supply. Foaming was reduced when halfstrength MS medium minerals were used (Ziv 1995a) and by lowering the concentration of calcium in the medium (Takayama 1991). Disposable plastic bioreactors with a volume of 2 and 5-l capacity used for organogenic micropropagation were found to provide good circulation with reduced cell shearing, cell damage and foaming. Meristematic and bud clusters are less shear-sensitive than large, vacuolated cells (Ziv et al., 1998).

The excessive accumulation of water in plant tissue (the most characteristic symptom of hyperhydricity) can result in oxygen depletion in the cells, induce oxidative stress, production of reactive oxygen species (ROS) and cause injury to the plant tissue. Several developmental processes in tissue cultured plants can be affected by ROS leading to recalcitrance and loss of morphogenetic competence (Benson, 2000). ROS such as H_2O_2 and hydroxyl free-radicals react instantaneously with almost any substrates (Levine, 1999).

In *Narcissus* liquid cultures, antioxidant enzyme activities were found to correlate with hyperhydric shoots and leaf section explants malformation (Chen and Ziv, 2001). In ancymidol (ANC) treated hyperhydric *Narcissus* shoots, ascorbate peroxidase (APX) and catalase (CAT) activities were significantly greater than in their non-treated, non-hyperhydric counterparts. In the ANC-treated hyperhydric Narcissus leaf sections, APX and CAT activities were significantly less than in the non-hyperhydric ones cultured in medium lacking ANC, especially during the period of meristematic centre formation. The formation of meristematic centres on ANC-treated hyperhydric leaf sections during the 3rd and 4th weeks in culture, could have resulted from lower H_2O_2 concentrations than in nontreated ones. Lower concentrations of H_2O_2 in ANC-treated hyperhydric leaf sections may cause a lower hydroxyl free-radical accumulation and therefore ANC-treated leaf sections may have the ability to develop meristematic centers. In root suspension cultures of *Hypericum perforatum* L (Cui et al., 2010c) higher sucrose concentrations increased the levels of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and proline indicating osmotic stress.

1.2.5 CASE STUDIES OF THE USE OF BIOREACTORS IN PLANTS

1.2.5.1 Types of propagules produced in the bioreactor

Many plant species and varieties have been cultured in the bioreactor. Responses of cultures in bioreactors are quite different among species or genera and they could be also different from the responses observed under static culture conditions on semi-solid medium. Various types of plant propagules such as shoots, bulbs, microtubers, corms and embryos have been successfully propagated in bioreactors. Several examples are as follows (Takayama and Akita, 2005; Takayama and Akita, 2006)

a) **Shoots**: *Atropa belldona, Begonia x hiemalis, Chrysanthemum morifolium, Dianthus caryophyllus, Fragaria ananassa, Nicotiana tabacum, Petunia hybrida, Primula obconica, Zoysia japonica, Scopolia japonica, Spathiphyllum, Stevia rebaudiana,* etc

b) **Bulbs**: *Lilium* species*, Fritillaria thunbergii, Hippeastrum hybridum, Gladiolus, Hyacinthus orientalis*.

c) **Corms:** *Colocasia esculenta, Pinellia ternata, Caladium* sp.

d) **Tubers:** *Solanum tuberosum*

e) **Embryos or adventitious buds**: *Atropa belladonna*

The propagules produced in the bioreactor should be easily to reestablished in soil, with as little adaptation to *ex vitro* conditions as possible. Storage organs such as bulbs, corms or tubers seem to be the best choice for proliferation in bioreactors.

1.2.5.2 Plant species propagated in bioreactors

The application for plant propagation was first reported in 1981 for *Begonia* (Takayama and Misawa, 1981). The techniques have been applied to many plant species since then

- **Potato tubers** using jar fermentor techniques (Akita and Takayama, 1988)
- Somatic embryogenesis in bioreactor culture (Preil and Beck,1991)
- ***Lilium longiflorum*** - Micropropagation of virus free bulblets by tank culure (Takahashi et al., 1992)
- ***Astragalus membranaceus*** - Bioreactor application on adventitious root culture (Wu et al., 2011)

- **Potato tubers** propagated in a jar fermentor (Akita and Takayama, 1993a,b), (Akita and Takayama, 1994a)
- ***Solanum tuberosum*** by semicontinuous liquid medium (Akita and Takayama, 1994b)
- **Types of bioreactors used for shoots and embryos** (Takayama and Akita, 1994)
- ***Picea mariana*** **and** ***Picea glauca*****-engelmannii** - Maturation of somatic embryo cultures (Tautorus et al., 1994)
- ***Nerine*** - Scaled-up proliferation and regeneration in liquid cultures (Ziv et al., 1994)
- ***Nerine*** - Somatic embryos and bulblet development from bioreactor regenerated meristematic clusters (Ziv et al., 1995b)
- **Potato bud clusters** - Propagation and tuberization from bioreactor culture (Ziv and Shemesh, 1996)
- ***Capsicum annuum*** -Somatic embryogenesis (Mavituna and Buyukalaca, 1996)
- ***Oryza sativa*** –Effect of oxygen-enriched aerationon regeneration in cell culture (Okamoto, 1996)
- **Sugarcane** in an improved temporary immersion system (Lorenzo et al., 1998)
- ***Colocasia esculenta*** in large scale without forced aeration (Akita and Ohta, 1996)
- ***Solanum tuberosum*** **L** using a bioreactor without forced aeration (Akita and Ohta, 1998)
- **Potato tubers** in a nutrient mist bioreactor (Hao et al., 1998)
- ***Ananas comosus*** - Micropropagation in temporary immersion systems (Escalona et al., 1999)
- ***Morinda elliptica*** cell suspension culture in a stirred-tank bioreactor (Abdullah et al., 2000)
- ***Dianthus caryophyllus*** **L**. - Grown in a mist reactor (Correll et al. 2000)
- ***Carthamus tinctorius*** yellow and red pigment production by cell cultures in a bioreactor (Gao et al., 2000)
- ***Picea sitchensis*** - Effect of bioreactor configuration on the growth and maturation (Ingram and Mavituna, 2000)
- ***Populus tremula*** **L** (roots) - Enhanced bud regeneration in liquid media (Vinocur et al., 2000)

- ***Lilium*** - Growth bulblet and formation from bulbscale segments using bioreactor system (Lian et at., 2003a,b)
- ***Chrysanthemum* shoots** - Multiplication in bioreactors as affected by culture method and inoculation density (Hahn and Paek, 2005)
- ***Spathiphyllum cannifolium*** - Mass propagation using an airlift bioreactor (Dewir et al., 2006)
- **Apple** – Micropropagation and nutrient utilization in temporary versus continuous immersion bioreactors (Chakrabarty et al., 2003, 2007)
- ***Hypericum perforatum*** Adventitious root- Biomass and secondary metabolites in bioreactor culture (Cui et al., 2010 a, b)
- Oncidium-Sugar Sweet- Production of protocorm like bodies with bioreactor (Yang et al., 2010)
- ***Rhodiola crenulata* shoots** - Mass multiplication using temporary immersion bioreactor (Zhao et al., 2012)
- ***Echinacea angustifolia*** Scale-up of adventitious root cultures in a pilot-scale bioreactor (Cui et al., 2012)
- ***Gypsophila paniculata*** - Improved micropropagation with bioreactor (Wang et al., 2013)

1.3 PLANT TISSUE CULTURE OF *LILIUM*

1.3.1 Propagation of *Lilium* by Bioreactor culture techniques

Lilium species are important flowering plants for bulb and cut flower production. The first investigations of propagation of *Lilium* in bioreactor culture were made using bulbscale segments as inocula, which resulted in the production of a large number of newly formed bulblets (Tsumaki and Takayama, 1992). Although the efficiency of the production of bulblets in liquid medium (shake or bioreactor) was different between species, almost the same culture conditions were optimal within the same species or genera. However, sometimes quite different culture conditions were optimal in different strains within same species of *L. auratum* (Takayama and Okuyama, 1996).

To achieve virus-free propagules Takahashi et al (1992) reported cultivation of *Lilium* bulblets in a 2000-liter bioreactor. In later studies Seon et al (2000) adapted a two-stage culture method in bulblet culture for *Lilium*: the first stage is for multiplication of bulblets from chopped bulb scale segments using an ebb and flood bioreactor; the second stage is for promoting the growth of bulblets using continuously immersed bioreactors. Initially, nitrogen uptake by scale segment was slow but almost all ammonium nitrogen in the culture medium was consumed after 5 wk of culture, while a large amount of nitrate nitrogen remained until 8 wk. Sucrose consumption was also completed in the early stage of culture as in the case of ammonium nitrogen. In this respect, ammonium nitrogen and sucrose were considered to play important roles in the bulblet formation. The uptake of other nutrients such as chloride, magnesium, potassium and calcium, with the exception of sulfate ion, were relatively low throughout the culture period. Sucrose was converted to glucose and fructose within 2 wk, which were equally consumed by the bulblets. The maximum value of DO in liquid is about 8 mg l^{-1} at saturation; thus, bulblets cultured in liquid medium have insufficient oxygen supply (compared to bulblets cultured on solid medium). Under a high DO concentration of 16 mgl^{-1}, phenolic compounds are released from cultured bulblets into the medium, resulting in slow bulblet growth. The pH decreased more rapidly than other factors such as DO concentration and sugar content (Seon et al., 2000). Bulblet growth at the second stage was influenced greatly by the sucrose concentration in the culture medium. In addition, bulblet growth was influenced by the types of bioreactor, showing over 10-fold growth in non-stirred jar, airlift column, and airlift balloon-type bioreactors after 2 months of culture compared to a stirred impeller-type bioreactor. The stirred impeller-type bioreactors inhibited the bulblet growth and eventually resulted in death of bulblets due to shear stress. The periodic immersion of bulblets using an ebb and flood system was employed in BTBB. After 3 months, bulblet circumference reached 5±6 cm, and more than 98% of bulblets sprouted when they were transferred to soil. This method is regarded as simple and cost-reductive as compared to conventional tissue culture methods, since no regular subculturing steps are required.

Lim et al. (1998) and Kim et al. (2001) reported a two-stage culture process of *Lilium* micropropagation (bulblet formation and then their development) in 5–20 l batches of non-stirred bioreactors within 60 days. Lian et al. (2003a) developed a more efficient two-stage bioreactor culture of *Lilium* bulblets using BTBB. In the first stage bulblets were induced from chopped bulb scales using an ebb and flood bioreactor. Although the

percentage of bulblet formation was lower in ebb and flood system as compared to gel-based culture, nevertheless were harvested a large number of bulblets from each batch culture. This bulblet culture in bioreactors (ebb and flood) will reduce the labour manipulations required for gel-based culture and facilitate scaling-up of bulblet production. The second stage is to promote the growth using continuous immersion bioreactors. Bulblets of *Lilium* Oriental Hybrid 'Casablanca' grew at a faster rate when the medium was exchanged with new medium frequently in a BTBB (immersion type) (Lian et al., 2003b). Uptake of sugar and other minerals indicate that high sucrose concentration are necessary for optimal *Lilium* bulblet growth. Although high sucrose concentrations could be maintained by the exchange method, the sucrose supplied was rapidly hydrolyzed into glucose and fructose when medium was replaced with new medium every 2, 6 and 12 weeks of the bioreactor culture. Mineral absorption also displayed variation, both in quantity and selectivity of the organic nutrients supplied. During the growth of bulblets, fast exhaustion of NH_4^+, NO_3^-, SO_4^{2-} and H_2PO^{4-} occurred, whereas consumption of K^+, Mg^{2+}, Ca^{2+}, Na^+ and Cl^- was slow. There was also a rapid decrease in pH of the medium following the addition of, or exchange with, fresh medium during the *Lilium* bulblet growth (Lian et al., 2002).

Ho et al. (2006) conducted a study with *Lilium X formolongi* Hort. cvs. Norikula, RaiZen No. 1, RaiZen No. 3, RaiZen Early, and Bailansa. *In vitro* seedlings of *Lilium x formolongi* Hort. Cvs were used to induce callus by variously modified Murashige and Skoog (MS) media, using protocols for flask culture and bioreactor culture. Results showed that the best cell growth and regeneration rate (74 ± 0.14%) of somatic embryos was in a modified 2-l bioreactor.

1.4 PLANT TISSUE CULTURE OF *Aloe barbadensis*

Aloe barbadensis syn vera. belongs to the family Liliaceae (Anonymous 1985). It is commonly called as 'Burn plant'. It is a xerophyte and can be grown even in dry lands under rain fed conditions. *Aloe* is a coarse looking perennial plant with a short stem, found in the semi-wild state in many parts of the country. Commercial *Aloe*s are obtained from wild as well as cultivated plants. Propagation is primarily by means of suckers (or) offshoots, which are separated carefully from mature plants and transplanted. Medium sized suckers are chosen and carefully dugout without damaging

the parent plant at the base and can be directly planted in the field. Plants will produce a commercial crop in one year (Venkataramaiah, 2003).

Aloe vera have been cultured *in vitro* by various researchers like Sanchez et al. (1988), Natali et al. (1990), Meyer and Staden (1991), Roy and Sarkar (1991), Corneanu et al. (1994). Richwine et al. (1995), Abrie and staden (2001), Chaudhuri and Mukandan (2001), Baksha el al. (2005), Adelberg and Naylor-Adelberg (2012), (Borgognone et al, 2013).

Sanchez et al. (1988) performed micropropagation from shoot meristems. They found that *in vitro* culture of *Aloe barbadensis* is very difficult for both callus induction and plant regeneration. A DNA microdensitometric study was performed on different organs *of A.barbadensis* and during *in vitro* culture of different explants. Natali et al. (1990) reported a rapid and highly effective plant micropropagation from vegetative meristems. Micropropagation was achieved by culturing shoot apices on medium containing 2,4-D and Kn within 15-30 days. High morphogenetic ability was maintained by transferring explants on media containing 2,4-D and 6-benzylaminopurine. (Natali et al., 1990), *Aloe*s have been cultured *in vitro* with 2,4-D and cytokinins in the growth medium (Groenewald *et al.* 1975, Natali *et al* 1990). Axillary bud development and adventitious bud formation was obtained with decapitated shoot explants of *Aloe barbadensis* Mill. Maximal bud growth and rooting of shoots was obtained on a modified medium of Murashige and Skoog supplemented with IBA. The optimal temperature for bud growth and development was 25 ^{0}C. Bud growth was slowed at 100c (Meyer and Staden, 1991). *Aloe* has also cultured for their high medicinal and ornamental value (Vij et al., 1980).

Little work has been done on callus culture of *Aloe* species because establishment of primary cultures is difficult owing to the secretion of the phenolic substances by explant. There is only one report on callus formation and plant regeneration from seed calli of *Aloe pretoriensis* (Groenewald et al., 1975). Roy and Sarkar (1991) report the rapid propagation by the formation of shoots from calli of *Aloe vera*. They induced the callus formation in stem segments from young axillary shoots grown on the underground rhizomatous stem. Polyvinylpyrrolidone was used to reduce the secretion of phenolic substances from the explant. Modified MS media with 2,4-D and Kinetin was used for callus induction. (Roy and Sarkar, 1991).

Micropropagation of *Aloe* was carried out by culturing fragments from axillary shoots on MS medium without growth regulators (the presence of which was found to inhibit the first stage of development). For the second stage of development involving the newly formed plantlets, the presence of a magnetic fluid in the culture medium stimulated secondary shoot production, general plant development and rhizogenesis (Corneanu et al., 1994). Richwine et al. (1995) reported the induction of shoot cultures of *Aloe, Gasteria* and *Haworthia* species from immature inflorescence. Shoots were initiated on a modified MS medium containing zeatin and later maintained on medium containing Zeatin and BA.

A rapid propagation protocol was established for the highly endangered *A. polyphylla*. Seed was germinated *in vitro* on MS medium with or without sucrose. Plantlets were cultured on medium containing benzyl adenine (BA) only, or a combination of BA and NAA. After initial problems with browning, the explants rapidly formed axillary and adventitious buds (Abrie and staden, 2001). Chaudhuri and Mukandan (2001) and Baksha et al. (2005), also reported that in *Aloe vera* formation of multiple shoots *in vitro* was a function of cytokinin and auxin concentrations. The presence of only cytokinin or auxin resulted in formation of callus or roots, best multiplication of shoots was obtained on medium containing BA + Adenine sulphate + IAA (Chaudhuri and Mukandan, 2001), while Baksha et al. (2005) founded that MS supplemented with BAP (2.0 mg/l) and NAA (0.5 mg/1), was the best medium for In vitro Culture of Shoot tip explants.

Adelberg and Naylor-Adelberg (2012) micropropagated shoots tips *of A. barbadensis* on agar and liquid medium at varied benzyladenine (BA) and meta-topolin (MT) concentrations (0, 1, 3.2, and 10 μM) for three successive culture cycles and then transferred to a greenhouse for growth. The authors showed that MT induced multiplication at the highest concentration (10 μM) and BA produced the greatest number of plantlets (at 3.2 μM) with optimal multiplication at approximately 6 μM. Liquid medium did not affect multiplication rate when compared with agar, but plants were twice as large from liquid as compared with those from agar at the time of transfer to the greenhouse. After five weeks of growth, plants in the greenhouse micropropagated on liquid culture were still larger than plants micropropagated on agar with BA and MT. A carryover of cytokinin inhibited rooting, and plants on agar were more severely affected than plants on the liquid medium. Using liquid medium led to

larger plants and lessened the cytokinin carryover effect on rooting without affecting the multiplication rate. Approximately 6 μM BA in liquid medium would be optimal for multiplication and rooting of *A. barbadensis* (Adelberg and Naylor-Adelberg, 2012)

1.5 REFERENCES

Abdullah MA, Ariff AB, Marziah M, Ali AM, Lajis NH (2000) Strategies to overcome foaming and wall-growth during the cultivation of Morinda elliptica cell suspension culture in a stirred-tank bioreactor. Plant Cell, Tissue and Organ Culture 60: 205-212

Adelberg J, Fári MG (2010) Applied physiology and practical bioreactors for plant propagation. Propagation of Ornamental Plants: 10:205-219

Aitken-Christie J, Jones C (1987) Towards automation: radiata pine shoot hedges in vitro. Plant Cell, Tiss. Org. Cult. 8: 185-196

Aitken-Christe J, Davies HE (1988) Development of a semi-automated micropropagation system. Acta Hort. 230: 81-87

Aitken-Christie J (1991) Automation. In: Debergh PC, Zimmerman RH. (Eds). Micropropagation. Dordrecht: Kluwer Academic Publishers, pp 342-354.

Akita M, Takayama S (1988) Mass propagation of potato tubers using jar fermentor techniques. Acta Hortic. 230: 55-62

Akita M, Ohta Y (1996) Development of a system for mass propagation of Colocasia esculenta in large scale without forced aeration. Acta Hort. 440: 554-559

Akita M, Ohta Y (1998) A simple method for mass propagation of potato (Solanum tuberosum L.) using a bioreactor without forced aeration. Plant Cell Reports 18: 284-287

Akita M, Takayama S (1993a) Resting periods and the field performance of potato tubers propagated in a jar fermentor. Plant Tissue Culture Letters 10: 255-259

Akita M, Takayama S (1993b) Effects of medium surface level control on the mass propagation of potato tubers using jar fermentor culture techniques. Plant Tissue Culture Letters 10: 242-248

Akita M, Takayama S (1994a) Induction and development of potato tubers in a jar fermentor. Plant Cell, Tiss. Org. Cult. 36: 177-182

Akita M, Takayama S (1994b) Stimulation of potato (Solanum tuberosum L.) tuberization by semicontinuous liquid medium surface level control. Plant Cell Reports 13: 184-187

Altman A, Loberant B (2000) Micropropagation of plants, principles and practices. In: Spier RE, Griffiths B, Scragg AH (Eds). The Encyclopaedia of Cell Technology. ISBN: 0-471-16123-3, John Wiley & Sons, Inc, New York, pp. 916-929

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion on explants. Plant Cell, Tiss. Org. Cult. 32: 55-60

Alper Y, Young R, Adelberg J, Rhodes B (1994) Mass handling of watermelon microcuttings. Trans-actions of the American Sociesty for Agricultural Engineering. 37: 1337-1343

Ammirato PV, Styer DJ (1985) Strategies for large scale manipulation of somatic embryos in suspension culture. In: Zaitlin M, Day P, Hollaender A (Eds). Biotechnology in Plant Science: Relevance to Agriculture in the Eigthies Academic Press, New York, pp. 161-178

Archambault J, Lvoie L, Villiams RD, Chavarie C (1995) Nutritional aspects of Daucus carota somatic embryo cultures performed in bioreactors. In: Terzi M (Ed) Current Issues in Plant Molecularand Cellular Biology.Kluwer Academic Publishers, Dordrecht, pp. 681–687

Azechi S, Hashimoto T, Murata A, Miyakita I, Ishii H (1985) Dynamic measurement of volumetric oxygen transfer coefficient. Jpn Tobacco Cult. Pulb. Crop. 20:73-85

Benson E (2000) Do free radicals have role in plant tissue culture recalcitrance? Special symposium: in vitro plant recalcitrance. In Vitro Cell Biol. 36: 163–170

Berthouly M, Etienne H (2005) Temporary immersion system: a new concept for use liquid medium in mass propagation. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp. 165-195

D. Borgognone, A. Marcucci, M. Renzaglia, M. Cardarelli (2013). In vitro growth of Aloe barbadensis Mill.: the effect of activated charcoal on medium pH, nitrogen uptake and elements content of shoots. Propagation of Ornamental Plants 13 (3): 138-145.

Cabasson C, Alvard D, Dambier D, Ollitrault P, Teisson C (1997) Improvement of Citrus somatic embryo development by temporary immersion. Plant Cell. Tiss. Org. Cult. 50: 33- 37

Chakrabarty D, Hahn EJ, Yoon YS, Paek KY (2003) Micropropagation of apple root stock 'M9 EMLA' using bioreactor. J Hortic Sci Biotechnol 78:605–609

Chakrabarty D, Dewir YH, Hahn EJ, Datta SK, Paek KY (2007) The dynamics of nutrient utilization and growth of apple root stock 'M9 EMLA' in temporary versus continuous immersion bioreactors. Plant Growth Regul 51: 11–19

Chen J, Ziv M (2001) The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid-cultured Narcissus. Plant Cell Rep. 20: 22–27

Coombs J (1986) MacMillan Dictionary of Biotechnology. Macmillan Press, London, 330 pp.

Correll MJ, Wu Y, Weathers PJ (2000) Controlling hyperhydration of carnations (Dianthus caryophyllus L.) grown in a mist reactor. Biotechnol. Bioeng. 71: 307-314

Cui HY, Baque MA, Lee EJ, Paek KY (2012) Scale-up of adventitious root cultures of Echinacea angustifolia in a pilot-scale bioreactor for the production of biomass and caffeic acid derivatives. Plant Biotechnol Rep. Online ISSN 1863-5474

Cui XH, Chakrabarty D, Lee EJ, Paek KY (2010a) Production of adventitious roots and secondary metabolites by Hypericum perforatum L. in a bioreactor. Bioresource Technology 101: 4708–4716

Cui XH, Murthy HN, Wu CH, Paek KY (2010b) Adventitious root suspension cultures of Hypericum perforatum: effect of nitrogen source on production of biomass and secondary metabolites. In Vitro Cell.Dev.Biol. Plant 46:437–444

Cui XH, Murthy HN, Wu CH, Paek KY (2010c) Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of Hypericum perforatum L. Plant Cell Tiss Organ Cult. 103:7–14

Dantas AK, Majada JP, Fernández B, Cañal MJ (2001) Mineral nutrition in carnation tissue cultures under different ventilation conditions. Plant Growth Regulation. 33:237-243

Denchev PD, Kuklin AI, Scragg AH (1992) Somatic embryo production in bioreactors. J. Biotechnology. 26: 99-109

Dewir HY, Chakrabarty D, Hahn EJ, Paek KY (2006) A simple method for mass propagation of Spathiphyllum cannifolium using an airlift bioreactor. Vitro Cell. Dev. Biol Plant. 42:291–297

Dussert S, Verdeil JL, Rival A, Noirot M, Morel JB (1995) Nutrient uptake and growth of in vitro coconut (Cocos nucifera L.) calluses. Plant Sci. 106: 185–193

Escalant JV, Teisson C, Cote F (1994) Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivars (Musa spp.). In Vitro Cell. Dev. Biol. 30: 181-186

Escalona M, Lorenzo JC, González B, Daquinta M, Fundora Z, Borroto CG, Espinosa D, Arias E, Aspiolea ME (1998) New system for in vitro propagation of pineapple (Ananas comosus L. Merr). Pineapple News 5: 5-7

Escalona M, Lorenzo JC, Gonzalez B, Daquinta M, Gonzalez JL, Desjardins Y, Borroto CG (1999) Pineapple (Ananas comosus L. Merr) micropropagation in temporary immersion systems. Plant Cell Reports 18: 743-748

Etienne-Barry D, Bertrand B, Vasquez N, Etienne H (1999) Direct sowing of Coffea arabica somatic embryos mass-produced in a bioreactor and regeneration of plants. Plant Cell Rep. 19: 111-117

Etienne H, Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell, Tiss. Org. Cult. 69: 215-231

Etienne H, Bertrand B, Anthony F, Côte F, Berthouly M (1997a) L'embryogenèse somatique: un outil pour l'amélioration génétique du caféier. In: ASIC Publishers (eds) 17th International Scientific Colloquiun on Coffee, Nairobi, Kenya. Vevey, Switzerland, pp. 457-465

Etienne H, Lartaud M, Michaux-Ferriere N, Carron MP, Berthouly M, Teisson C (1997b) Improvement of somatic embryogenesis in Hevea brasiliensis (Müll. Arg.) using the temporary immersion technique. In Vitro Cell. Dev. Biol. 33: 81-87

Gao WY, Fan L, Paek KY (2000) Yellow and red pigment production by cell cultures of Carthamus tinctorius in a bioreactor. Plant Cell, Tiss. Org. Cult. 60: 95-100

Gawel NJ, Robacker CD (1990) Somatic embryogenesis in two Gossypium hirsutum genotypes on semi-solid versus liquid proliferation media. Plant Cell, Tiss. Org. Cult. 23: 201-204

George E, Hall M, De Klerck GJ (2008) Plant propagation by tissue culture. Springer, Dordecht, The Netherlands, 134p

Gómez K R, Vilchez P J, Albany VN, Agramonte PD (2005) Somatic embryo germination of Psidium guajava L. in the RITA® temporary immersion system and on semisolid medium. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp. 225-229

Grigoriadou K, Vasilakakis M, Tzoulis T, Eleftheriou EP (2005) Experimental use of a novel temporary immersion system for liquid culture of olive microshoots. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp. 263–274.

Hahn EJ, Paek KY (2005) Multiplication of Chrysanthemum shoots in bioreactors as affected by culture method and inoculation density of single node stems. Plant Cell, Tissue and Organ Culture. 81: 301–306

Hammerschlag F (1982) Factors affecting establishment and growth of peach shoots in vitro. HortScience 17: 85-86

Hao Z, Ouyang F, Geng Y, Deng X, Hu Z, Chen Z (1998) Propagation of potato tubers in a nutrient mist bioreactor, Biotechnology Techniques 12: 641-644

Harris RE, Mason EBB (1983) Two machines for in vitro propagation of plants in liquid media. Can. J. Plant Sci. 63: 311-316

Hempfling T, Preil W (2005) Application of a temporary immersion system in mass propagation of Phalaenopsis. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp. 231–242.

Heyerdahl PH, Olsen OAS, Hvoslef-Eide AK (1995) Engineering aspects of plant propagation in bioreactors. In: Aitken-Christie J, Kozai T, Smith L M (Eds) Automation and Environmetal Control in Plant Tiss. Cult. Kluwer Academic Publishers, Dordrecht, pp. 87-123

Hilton MG, Wilson PDG (1995) Growth and the uptake of sucrose and mineral ions by transformed root cultures of Datura
stramonium, Datura candida aurea wrightii, Hyoscyamus muticus and Atropa belladonna. Planta Medica 61: 345–350

Ho CW, Jian WT, Lai HC (2006) Plant regeneration via somatic embryogenesis from suspension cell cultures of Lilium x formolongi hort. using a bioreactor system. In Vitro Cell. Dev. Biol. Plant 42:240–246.

Honda H, Liu C, Kobayashi T (2001) Large-scale plant micropropagation. Adv. Biochem. Eng. Biotechnol. 72: 157-182

Hooker BS, Lee JM (1990) Cultivation of plant cells in a stirred vessel: effect of impeller design. Biotechnol. Bioeng. 35: 296-304

Ingram B, Mavituna F (2000) Effect of bioreactor configuration on the growth and maturation of Picea sitchensis somatic embryo cultures. Plant Cell, Tiss.Org. Cult. 61: 87- 96

Jay V, Genestier S, Courduroux JC (1992) Bioreactor studies on the effect of dissolved oxygen concentrations on growth and differentiation of carrot (Daucus carota L.) cell cultures. Plant Cell Rep. 11: 605-608

Jeong JA, Wu CH, Murthy HN, Hahn EJ, Paek KY (2009) Application of an Airlift Bioreactor System for the Production of Adventitious Root Biomass and Caffeic Acid Derivatives of Echinacea purpurea. Biotechnology and Bioprocess Engineering, 14: 91-98

Jimenez E, Perez N, de Feria M, Barbon R, Capote A, Chavez M, Quiala E, Perez JC (1999) Improved production of potato microtubers using a temporary immersion system. Plant Cell, Tiss. Org. Cult. 59: 19-23

Jones AM, Petolino JF (1988) Effects of support medium on embryo and plant production from cultured anthers of soft-red winter wheat. Plant Cell, Tiss. Org. Cult. 12: 243-261

Kim YS, Hahn EJ, Paek KY (2001) A large scale production of Lilium bulblets through bioreactor culture. Acta Hort. 560: 383–386

Leathers RR, Smith MAL, Aitken-Christie J (1995) Automation of the bioreactor process for mass propagation and secondary metabolism. In: Aiken-Christie J, Kozai T, Smith MAL (Eds). Automation and environmental control in plant tissue culture. Dordrecht: Kluwer Academic Publishers, pp. 187-214

Lee YH (1997) Design of bioreactor system for small and pilot scale cultivation of plant cells. MSc Thesis, Chungbuk National University, Korea

Levine A (1999) Oxidative stress as a regulator of environmental responses in plants. In: Lerner HR (Ed) Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization. Marcel Dekker Inc., NY, pp. 247–264

Lian M, Chakrabarty D, Paek KY (2002) Growth and uptake of sucrose and mineral ions by bulblets of Lilium Oriental Hybrid 'Casablanca' during bioreactor culture. J. Hortic. Sci. Biotechnol. 77: 253–257

Lian M, Chakrabarty D, Paek KY (2003a) Bulblet formation from bulbscale segments of Lilium using bioreactor system, Biologia Plantarum. 46: 199-2003

Lian ML, Chakrabarty D, Paek, KY (2003b) Growth of Lilium Oriental Hybrid ‘Casablanca’ bulblet using bioreactor culture. Scientia Hort. 97: 41-48

Lim S, Seon JH, Paek KY, Son SH (1998) Development of pilot scale process for mass production of Lilium bulblets in vitro. In: Drew RA (Ed) Proc. Int. Symp. Biotechnology Tropical and Subtropical Species Acta Horti. pp. 237–241

Lorenzo JC, Boris LG, Escalona M, Teisson C, Espinosa P, Borroto C (1998) Sugarcane shoot formation in an improved temporary immersion system Plant Cell, Tissue and Organ Culture 54: 197–200

Luna C, Collavino M, Sansberro P, Mroginski L (2009) Bacterial contamination in Ilex dumosa (aquifoliaceae) cultures: antibiotic treatment. Acta Hort. 812: 97-102

Maene L, Debergh P (1985) Liquid medium additions to established tissue cultures to improve elongation and rooting in vivo. Plant Cell, Tiss. Org. Cult. 5: 23-33

Mavituna F, Buyukalaca S (1996) Somatic embryogenesis of pepper in bioreactors: a study of bioreactor type and oxygen-uptake rates. Appl Microbiol Biotechnol 46:327-333

Okamoto A, Kishine S, Hirosawa T, Nakazono A (1996) Effect of oxygen-enriched aerationon regeneration of rice (Oryza sativa L.) cell culture. Plant Cell Reports 15: 731-736

Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor system for large scale production of horticultural and medicinal plants. Plant Cell, Tissue and Organ Culture. 81: 287-300

Paek KY, Hahn EJ, Son SH (2001) Application of bioreactors for large-scale micropropagation systems of plants. In Vitro Cellular and Development Biology – Plant. 37: 149-157

Preil W (1991) Application of bioreactors in plant propagation. In: Debergh PC, Zimmerman RH (Eds). Micropropagation Technology and Application. Kluwer Academic Publishers, Dordrecht, pp. 425-446

Preil W (2005) General introduction: a personal reflection on the use of liquid media for in vitro culture. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp. 1-18

Preil W, Beck A (1991) Somatic embryogenesis in bioreactor culture Acta Hortic. 289: 179-192

Preil W, Florek P, Wix U, Beck A (1988) Towards mass propagation by use of bioreactors. Acta Hort. 226: 99-105

Sajc L, Grubisic D, Novakovic GV (2000) Bioreactors for plant engineering: An out for further research. Biochem. Eng. 4: 89–99

Schiigerl K, Sittig W (1987) Bioreactors. In: Preile P, Faust U, Sittig W, Sukatsch DA (Eds). Basic biotechnology. Weinheim: VCH Verlagsgesellschaft, pp. 179-224

Schmitz U, Lorz H (1990) Nutrient uptake in suspension culture of Gramineae. II. Suspension cultures of rice (Oryza sativa L). Plant Sci. 66: 95–111

Seon JH, Kim YS, Son SH, Paek KY (2000) The fed-batch culture system using bioreactor for the bulblet production of oriental lilies. Acta Hort. 520:53-59

Seon JH, Yoo KW, Cui YY, Kim MH, Lee SS, Son SH, Paek KY (1998) Application of bioreactor for the production of saponin by adventitious root cultures in Panax ginseng. In: Altman A, Ziv M, Izhar S (Eds). Plant biotechnology and in vitro biology in the 21st century. Dordrecht: Kluwer Academic Publishers, pp. 329-332

Shin KS, Chakrabarty D, Ko JY, Han SS, Paek KY (2003) Sucrose utilization and mineral nutrient uptake during hairy root growth of red beet (Beta vulgaris L.) in liquid culture. Plant Growth Regulation 39: 187–193

Simonton W, Robacker C, Krueger S (1991) A programmable micropropagation apparatus using cycled medium. Plant Cell, Tiss. Org. Cult. 27: 211-218

Son SH, Choi SM, Kwon SR, Lee YH, Paek KY (1999a) Large scale culture of plant cell and tissue by bioreactor system. J. Plant Biotechnol. 1: 1–8

Son SH, Choi SM, Lee DS, Yun SR, Paek KY (1999b) Commercial application of mountain ginseng through bioreactor culture system. In: Paek KY (Ed). Korea-Japan joint symposium on transplant production in horticultural plants. Res. Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Korea, pp. 9-14.

Steward FC, Caplin S, Millar FK (1952) Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. Ann. Bot. 16: 57-77

Steward FC, Shantz EM (1956) The chemical induction of growth in plant tissue cultures. In: Wain RL, Wightman F (Eds). The Chemistry and Mode of Action of Plant Growth Substances (Ed). Butterworths Ltd. London, pp. 165-186

Takahashi S, Matsubara K, Yamagata H, Morimoto T (1992) Micropropagation of virus free bulblets of Lilium longiflorum by tank culure, 1. Development of liquid culture method and large scale propagation. Acta Hortic. 319: 83-88

Takayama S (1991) Mass propagtion of plants through shake and bioreactor-culture techniques. In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry, Springer Verlag, Heidelberg, pp. 495- 515

Takayama S (2000) Bioreactors, Airlift. In: Spier RE, Griffiths B, Scragg AH (Eds). The Encyclopedia of Cell Technology. ISBN: 0-471-16123-3, John Wiley & Sons, Inc, New York, pp. 201-218

Takayama S, Akita M (1994) The types of bioreactors used for shoots and embryos. Plant Cell, Tiss. Org. Cult. 39: 147-156

Takayama S, Akita M (1998) Bioreactor techniques for large-scale culture of plant propagules. Adv. Hort. Sci. 12: 93-100

Takayama S, Akita M (2005) Practical aspects of bioreactor application in mass propagation of plants. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp. 1-18

Takayama S, Akita M (2006) Bioengineering aspects of bioreactor in plant propagation. In: Gupta SD, Ibaraki Y (Eds). Plant Tissue Culture Engineering. Springer, Dordrecht, The Netherlands, pp. 83-100

Takayama S, Misawa M (1981) Mass propagation of Begonia x hiemalis plantlets by shake culture. Plant Cell Physiol. 22: 461-467

Takayama S, Okuyama K (1996) Effects of the number of inoculated bulbscales and the culture strain on differentiation and growth of Lilium auratum in tissue culture. J. Soc. High Technol. Agric. 8: 175-180

Teisson C, Alvard D (1995) A new concept of plant in vitro cultivation liquid medium: Temporary immersion. In: Terzi M, Cella R, Falavigna A (Eds). Current Issues in Plant Molecular and Cellular Biology. Kluwer Academic Publishers, Dordrecht, pp. 105-110

Tisserat B, Vandercook CE (1985) Development of an automated plant culture system. Plant Cell, Tiss. Org. Cult. 5: 107-117

Tormala T, Jokinen K, Majamemi A, Tuominen U (1987) Utilization of media components by begonia shoots in submerged culture. In: Neijssel OM, Van der Meer RR, Luyben KCAM (Eds). Proc. 4th Europe Cong. Biotech. 2.Elsevier Science, Amsterdam, pp. 391–394

Tsumaki N, Akayama S (1992) Simple mass propagation of Lilium auratum by shake and jar fermentor culture. J. Soc. High Technol. Agric. 3: 124-128

Vinocur B, Carmi T, Altman A, Ziv M (2000) Enhanced bud regeneration in aspen (Populus tremula L.) roots cultured in liquid media. Plant Cell Reports 19: 1146-1154

Wang SM, Piao XC, Park SY, Lian ML (2013) Improved micropropagation of Gypsophila paniculata with bioreactor and factors affecting ex vitro rooting in microponic system. In Vitro Cell.Dev.Biol.—Plant. 49: 70-78

Wawrosch CH, Kongbangkerd A, Köpf A, Kopp B (2005) Shoot regeneration from nodules of Charybdis sp.: A comparison of semisolid, liquid and temporary immersion culture systems. In: Hvoslef-Eide AK, Preil W (Eds). Liquid Culture

Systems for in vitro Plant Propagation. Springer, Dordrecht, The Netherlands, pp275-280

Wu SQ, Lian ML, Gao Ri, Park SY, Piao XC (2011) Bioreactor application on adventitious root culture of Astragalus membranaceus. In Vitro Cellular & Developmental Biology - Plant 47: 719-724

Yang JF, Piao XC, Sun D, Lian ML (2010) Production of protocorm-like bodies with bioreactor and regeneration in vitro of Oncidium Sugar Sweet Scientia Hort. 125: 712-717

Yu KW, Gao WY, Hahn EJ, Paek KY (2001a) Effects of micro elements and nitrogen source on adventitious root growth and ginsenoside production in ginseng (Panax ginseng C. A. Meyer). J. Plant Biol. 44: 179–184

Yu KW, Gao WY, Hahn EJ, Paek KY (2001b) Effects of NH4 +:NO3- ratio and ionic strength on adventitious root growth and ginsenoside production in bioreactor culture of Panax ginseng C.A. Meyer. Acta Hort. 560: 259–261

Yu WC, Joyce PJ, Cameron DC, McCown BH (2000) Sucrose utilization during potato microtuber growth in bioreactors. Plant Cell Rep. 19: 407–413

Zhao Y, Sun W, Wang Y, Saxena PK, Liu CZ (2012) Improved mass multiplication of Rhodiola crenulata shoots using temporary immersion bioreactor with Forced Ventilation. Appl Biochem Biotechnol. 166:1480–1490

Ziv M (1992) The use of growth retardants for the regulation and acclimatization of in vitro plants. In: Karssen CM, Van Loon LC, Vreugdenhil D (Eds). Progress in Plant Growth Regulation. Kluwer Acad. Publ., Dordrecht, pp809-817

Ziv M (1995) The control of bioreactor environment for plant propagation in liquid culture. Acta Hortic. 393: 25-38

Ziv M (2000) Bioreactor technology for plant micropropagation. Horticultural Reviews 24: 1- 30

Ziv M (2005) Simple bioreactors for mass propagation of plants. Plant Cell, Tissue and Organ Culture. 81: 277–285

Ziv M, Hadar A (1991) Morphogenic pattern of Nephrolepsis exaltata Bostoniensis in agar-gelled or liquid culture. Implication for mass propagation. Israel J. Bot. 40: 7–16

Ziv M, Kahany S, Lilien-Kipnis H (1994) Scaled-up proliferation and regeneration of Nerine in liquid cultures: Part I. The induction and maintenance of proliferating meristematic clusters by paclobutrazol in bioreactors. Plant Cell, Tiss. Org. Cult. 39: 109- 115

Ziv M, Kahany S, Lilien-Kipnis H (1995) Somatic embryos and bulblet development from bioreactor regenerated meristematic clusters of Nerine. Acta Hortic. 393: 203-212

Ziv M, Ronen G, Raviv M (1998) Proliferation of meristematic clusters in disposable pre-sterilized plastic bioreactors for large-scale micropropagation of plants. In Vitro Cell Dev. Biol. Plant 34: 152–158

Ziv M, Shemesh D (1996) Propagation and tuberization of potato bud clusters from bioreactor culture. In Vitro Cellular and Developmental Biology Plant 32: 31-36

2 EXPERIMENTAL PART

2.1 Response to *In Vitro* Propagation of Different Asiatic hybrid Lilies

(Cardona Suárez, C.M., Colla, G., Cardarelli, M. and Nesi, B. 2011. Acta Hort. 900:393-399)

Keywords: bulblet regeneration, shoot formation, shoot clusters, explant size.

2.1.1 ABSTRACT

In vitro **bulb scales of seven Asiatic hybrid lily (*Lilium* x *elegans* Thunb) lines ('12', '20', '599', '409', '00144', '00150' and '00151'), differing for scale size (large and small), were cultured on MS medium supplemented with 8.8 µM BA and 1.1 µM NAA to induce adventitious shoots formation. After 6 weeks, the frequency of shoot formation was very high in almost all genotypes (>94%); new shoots were obtained from the basal part of bulb scales and became shoot clusters. These shoot clusters were cut into sections of about 4–7 mm in size at random and then cultured on the same medium for 6 weeks. During the first and second subculture period, bulblets formed on large explants were greater and formed more shoots than small ones. In the third subculture different combinations of BA (2.2 - 8.8 - 17.6 µM) and NAA (1.1 - 2.2 µM) were used to examine the effect on shoot cluster regeneration. The media supplemented with 2.2 µM BA or 8.8 µM BA and 2.2 µM NAA were the most effective to promote shoot proliferation, shoot length and root growth, moreover the medium supplemented with 8.8 µM BA and 2.2 µM NAA was most effective to increase the biomass. In all subcultures, the hybrids '00150', '00144' and '20' showed the highest values of shoot number, fresh weight, dry weight, shoot length and growth index. Going from first to third subculture, shoot cluster cycle method improved adventitious shoot formation and increased the biomass. All plantlets were successfully acclimated under greenhouse conditions.**

2.1.2 INTRODUCTION

The floriculture industry is constantly looking for product innovation i.e. new species or cultivars with interesting commercial traits, such as different flowering time,

plant size, flower colour, bulbils production and pollenless flowers (Grassotti et al., 2010).

Lily, a monocotyledonous species belonging to the Liliaceae, is one of the most important cut-flower species, mainly because of its large, attractive flowers. Lilies are usually propagated by scaling, a technique which produces 3–5 bulbs from each bulb scale, depending on species, cultivar and scale size (Han et al., 2004). Tissue culture is an alternative propagation method to produce a great number of high quality plantlets and to increase the plant growth rate allowing large-scale production of lily bulblets. Even if the i*n vitro* multiplication of lily has been reported from leaves, anthers, stems and tepals, the regeneration of bulblets from excised bulb scales is the preferred method for vegetative propagation of lilies (Lian et al., 2003). According to Langens-Gerrits et al. (2003), the scale size is one of the main factors that positively affects the *in vitro* bulb multiplication rate.

The aim of the present research was to describe a method for micropropagation of seven pollenless Asiatic hybrid lilies (L. x *elegans* Thunb) taking into consideration the genotype, the culture medium and the scale size. The pollenless Asiatic hybrid lilies were obtained in a intensive breeding program conducted at the CRA-VIV Experimental Institute for Floriculture, Pescia (Pistoia, Italy).

2.1.3 MATERIALS AND METHODS

Bulblets of Asiatic hybrid lilies, selection ‘12’, ‘20’, ‘599’, ‘409’, ‘00144’, ‘00150’ and ‘00151’, were provided by CRA-VIV, Pescia, Italy. Bulb scales segments, large (6 x 15 mm) and small (3 x 10 mm), were washed under running tap water for 10 min and then surface-sterilized by immersions in ethanol 70% (v/v, 1 minute) and sodium hypochlorite 2% of active chlorine (25 minutes), rinsed with sterile distilled water and cultured onto solidified base medium (0.6% agar), containing MS (Murashige and Skoog, 1962) salts and vitamins supplemented with 6% (w/v) sucrose, 8.8 µM benzylaminopurine (BA), 1.1 µM naphthalene acetic acid (NAA). The medium was adjusted to pH 5.8 and autoclaved for 15 min (at 121°C/103 kPa). Explants were individually placed in glass culture tubes containing 10 ml of medium and cultured at 25°C±1°C, under white fluorescent lights (40 µmol m^{-2} s^{-1}; 16-h photoperiod) (1st subculture). After six weeks shoot clusters obtained from the basal parts of bulb scale

segments were randomly cut into sections of about 4–7 mm in size, and then cultured on the same medium for other 6 weeks (2nd subculture). In the 3rd subculture, sections of bulb scale clusters (4-7 mm) were transferred to MS medium (0.6 % agar) (pH 5.8) with 6 % (w/v) sucrose added with different levels of BA (2.2 - 8.8 - 17.6 µM) and NAA (1.1 - 2.2 µM) to examine the effect on shoot cluster proliferation. The subcultures two and three were performed under the environmental conditione described above in 250 ml glass flasks containing 100 ml of culture medium. During the experiments, the following measurements were made: shoot formation (percentage of scales forming bulbs), number of shoots produced by each explant, length and diameter of shoots, number of roots. Growth index [GI = (weight final – weight initial)/weight initial] was calculated as described by Russowski et al. (2006). Bulblets fresh weight and dry weight was measured after drying at 80°C for 48 h. Bulblets with weight over 800 mg were washed in running tap water and soaked in fungicide, Topsin-M® (0.1 %, v/v) for a few minutes, after they were transferred to a sterile perlite:vermiculite (1:1, v/v) substrate in plastic pots for acclimatization. The pots were placed for 2 weeks at 25 ± 1°C in a plastic tunnel and then transferred in greenhouse.

Experiments were set up in a complete randomized design (10 replicates per treatment). Data were analyzed by ANOVA using the SPSS software package (SPSS 10 for Windows, 2001). Duncan's multiple range test was performed at P=0.05 on each of the significant variables measured.

2.1.4 RESULTS AND DISCUSSION

All the hybrids tested were able to differentiate new shoots in 6 weeks, from the basal part of both large and small bulb scales. At the end of the 1st subculture bulb scales produced shoot clusters. The percentage of shoot formation was very high in almost all genotypes (higher than 94%), except for hybrid "12" (Table 1). Significant differences among the hybrids were detected for shoot number, length and diameter of shoots, fresh and dry weight. A significant "hybrid x explants size " interaction was observed for shoot number per bulb scale (data not shown) with the highest value in hybrid "00150" (4.3). Hybrids "599" and "00150" accumulated more biomass in terms of fresh and dry weight, while the lowest values were obtained from "12" and "00151" hybrids. The size of the explanted bulb scale had a significant effect on shoot formation and their biometrical measures (Table 1). The large explants produced a major number of higher and larger shoots with a significant biomass accumulation capability. Explant size effect can be ascribed to two main factors: (i) large explants provide more reserves

for bulblets growth than small ones and (ii) sucrose, nutrients and hormones uptake is related with the cut surface contacting the medium which is obviously bigger in large explants than in the small ones (Langens-Gerrits et al., 2003). Our results agree with those reported by Langens-Gerrits et al. (2003) which found out that explants size affected Lilium bulb growth during the complete culture period and bulblets developed on large explants were bigger and accumulated higher amount of dry matter than those developed on the small-ones.

In the 2nd subculture each shoot cluster was considered as explant. Hybrids and bulb scale size were compared on the base of the multiplication rate and shoot biometrical characters (Table 2). The highest number of shoots/explant formed by "00150" and "00144" was statistical different from the lowest values performed by hybrids "12" and "00151". Hybrids "20" and "00150" accumulated more biomass than the other ones. Rooting results were not statistical different between hybrid "20" and "00144", "599", "00150" and "409" but they significantly differed from the limited rooting capability of hybrid "12" (Table 2). Since this rooting capability is lower than those reported by other authors on Lilium species (Han et al., 2004; Saifullah et al., 2010), probably there was an inhibitor effect of BA on roots formation as also observed by Han et al. (2004). The size of the starting explant continued to affect the shoots cluster performances independently from the hybrid, even if these effects were less significant than those observed at the end of the 1st subculture (Table 2).

In the 3rd subculture all hybrids were cultured with different concentrations of BA and NAA. The media supplemented with 2.2 μM or 8.8 μM BA and 2.2 μM NAA were the most effective treatments on promoting the proliferation of shoots (Table 3), as reported by other authors (Bacchetta et al., 2003; Nhut, 2003; Han et al., 2004; Han et al., 2005; Kapoor et al., 2009; Ishimori et al., 2009; Saifullah et al., 2010). Among the hybrids, the bulblet regeneration was statistically highest in hybrids "20", "00144" and "00150" while the lowest response was recorded in hybrids "12" and "00151". The interaction between hybrids and treatments on shoot/explant was statistically significant and revealed that the medium supplemented with 8.8 μM BA and 1.1 μM NAA produced highest number of shoots in hybrid "00150" (Table 3).

The highest fresh weight (Table 4) and grown index 3.6±0.43 (data not shown) were obtained on medium supplemented with 8.8 μM BA and 2.2 μM NAA; the interaction between hybrids and treatments on fresh weight was statistically significant

and revealed that highest values were achieved on medium supplemented whit 8.8 μM BA and 1.1 μM NAA in hybrids "00150 and "00144" (Table 4). This result indicates that average concentrations of cytokinins in combination with auxins are effective in increased the biomass (Kapoor et al., 2008).

The most effective treatment on promoting root formation was the medium supplemented with 2.2 μM BA and 2.2 μM NAA, while root formation was inhibited with 17.6 μM BA and 1.1 μM NAA (Table 5) (Nhut, 1998; Saifullah et at., 2010). The presence of NAA was also reported as prerequisite for the root formation in Narcissus bulbocodium (Santos et al., 1998) and Charybdis numidica (Kongbangkerd et al., 2005).

In Figure 1 is reported the growth index measured between subcultures two and three in hybrid lilies; the best results were obtained for hybrids "00150" and "00144" with values five times more than for other ones.

2.1.6 REFERENCES

Bacchetta, L.; Remotti, P.C.; Bernardini, C. and Saccardo, F. 2003.Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium*. Plant Cell, Tissue and Organ Culture 74: 37–44.

Grassotti, A., Nesi, S.; Lazzereschi, S.; Cacini, S. and Pacifi, S. 2010. New varieties of Asiatic Hybrids Lily: An Italian experience. Intl. Symposium on Genus *Lilium*. Pescia, Italy 30 Aug -.3 Sep. p. 80.

Han, B.H., Yu, H.J., Yae, B.W. and Peak, K.Y. 2004. *In vitro* micropropagation of *Lilium longiflorum* 'Georgia'by shoot formation as influenced by addition of liquid medium. J. Hort. Sci. 103: 39–49.

Han, B.H., Yae, B.W., Yu, H.J. and Peak, K.Y., 2005. Improvement of *in vitro* micropropagation of Lilium oriental hybrid 'Casablanca' by the formation of shoots with abnormally swollen basal plates. J. Hort. Sci 103: 351–359

Ishimori, T.; Niimi, Y. And Han, D.S. 2009. *In vitro* flowering of *Lilium rubellum* baker J. Hort. Sci 120: 246-249.

Kapoor, R.; Kumar, S. and Kanwara, J.K. 2008. Bulblet regeneration from *ex vit*ro root explants in lily hybrids. J. Hort. Sci. 35: 107-112

Kapoor, R.; Kumar, S. and Kanwara, J.K. 2009. Bulblet production from node explant grown *in vitro* in hybrid lilies. Int. J. Plant Production. 3: 1-6

Kongbangkerd, A.; Köpf, A.; Allacher, P.; Wawrosch, C. and Kopp, B. 2005. Micropropagation of squill (*Charybdis numidica*) through nodule cultura. Plant Cell Rep 23: 673-677.

Langens-Gerrits, M.; Kuijpers, A.M.; De Klerk, G.J. and Croes, A. 2003. Contribution of explant carbohydrate reserves and sucrose in the medium to bulb growth of lily regenerated on scale segments *in vitro*. Physiol. Plant. 117: 245–255.

Lian, M.L., Chakrabarty, D., and Paek, K.Y., 2003. Growth of *Lilium* Oriental Hybrid 'Casablanca' bulblet using bioreactor culture. Scientia Hort. 97: 41-48.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15: 473-497.

Nhut, D.T. 1998. Micropropagation of lily (*Lilium longiflorum*) via *in vitro* stem node and pseudo-bulblet cultura. J. Plant Cell Reports.17: 913–916

Nhut D.T. 2003. Plant growth regulation the control of *in vitro* direct main stem formation of *Lilium longiflorum* derived from receptacle culture, and rapid propagation by using *in vitro* stem nodes. Plant Growth Regulation 40: 179–184.

Russowski, D.; Maurmann, N.; Rech, S.B. and Fett-Neto, A.G. 2006. Role of light and medium composition on growth and valepotriate contents in *Valeriana glechomifolia* whole plant liquid cultures. Plant Cell Tiss Organ Cult. 86: 211-218.

Saifullah, K.; Sheeba, N.; Mariam, R.; Naheed, K.; Asma, N. and Bushra, S. 2010. Cultivation of lilies (*Lilium regale*) for commercialization in Pakistan. Pak. J. Bot., 42:1103-1113.

Santos, J.; Santos, I. and Salema, R.; 1998. *In vitro* production of bulbs of *Narcissus bulbocodium* flowering in the first season of growth. J. Hort. Sci 76:205-217

Tables

Table 1. Shoot formation from bulb scales of different hybrid lily lines after 6 weeks in culture on MS medium supplemented with 8.8 µM BA and 1.1 µM NAA (1th subculture).

Hybrids (lines)	Shoot formation (%)	Shoots/ bulb scale (n.)	Shoot length (mm)	Shoot diameter (mm)	Fresh wt./ bulb scale (mg)	Dry wt./ bulb scale (mg)
12	71.7 c[1]	2.6 bc	9.7 ab	4.9 ab	107.3 b	15.4 c
00151	88.1 b	1.9 d	4.0 c	4.1 b	84.5 b	14.7 c
00150	86.8 b	2.9 ab	7.6 bc	5.1 a	304.9 a	51.9 ab
00144	97.5 a	3.3 a	8.2 abc	5.1 a	241.5 ab	41.2 b
409	96.7 a	2.5 bc	9.1 ab	5.2 a	236.4 ab	40.3 b
599	98.2 a	2.9 ab	11.6 ab	5.4 a	357.5 a	60.8 a
20	96.8 a	2.5 bc	12.0 a	5.3 a	219.9 ab	43.2 b
Size explant						
Large (6-15mm)	96.2 a	3.2 a	11.1 a	5.4 a	331.3 a	57.1 a
Small (3-10mm)	85.5 b	2.2 b	6.6 b	4.7 b	112.1 b	19.4 b
Significance[2]						
Hybrids (H)	***	***	***	***	***	***
Size explant (SE)	*	***	**	**	***	***
H x SE	NS	**	NS	NS	NS	NS

[1]Means within columns separated using Duncan's multiple range test, P = 0.05

[2] NS, *, **, *** Non Significant or significant at P≤0.05, 0.01, and 0.001, respectively

Table 2. Shoot multiplication from segments of shoot clusters obtained *in vitro* in hybrid lilies after 6 weeks in culture on MS medium supplemented with 8.8 μM BA and 1.1 μM NAA (2nd subculture).

Hybrids (lines)	Shoots/ explant (n.)	Shoot length (mm)	Shoot diameter (mm)	Fresh wt./ explant (mg)	Dry wt./ explant (mg)	Roots/ explant (n.)
12	3.3 c[1]	22.8 b	5.3	534 cd	86 cd	0.9 b
00151	1.9 d	12.5 c	5.5	250 d	38 d	0.2 c
00150	5.4 a	18.2 bc	6.0	1124 b	212 b	2.1 ab
00144	5.2 ab	18.5 bc	5.9	992 b	182 bc	2.8 a
409	4.0 bc	20.2 bc	6.2	499 cd	87 cd	2.0 ab
599	4.0 bc	19.3 bc	6.3	856 bc	172 bc	2.7 a
20	4.5 abc	38.4 a	6.2	2235 a	515 a	3.0 a
Size explant						
Large (6-15mm)	4.9 a	22.3	6.2	1234 a	248 a	2.4 a
Small (3-10mm)	3.3 b	20.6	5.6	620 b	121 b	1.5 b
Significance[2]						
Hybrids (H)	**	***	NS	***	***	***
Size explant (SE)	**	NS	NS	*	*	*
H x SE	NS	NS	NS	NS	NS	NS

[1]Means within columns separated using Duncan's multiple range test, P = 0.05

[2] NS, *, **, *** Non significant or significant at P≤0.05, 0.01, and 0.001, respectively

Table 3. Effect of BA and NAA on number of shoots obtained from segments of shoot cluster in hybrid lilies after 6 weeks in culture [1].

Growth Regulators (μM)		Hybrid lines							**Mean**	
		12	00151	00150	00144	409	599	20		
BA	NAA									
2.2	1.1	2.0	1.3	3.9	3.3	2.4	3.1	3.5	3.0	b[2]
8.8	1.1	1.8	1.7	6.5	3.8	3.2	3.4	4.8	3.2	b
17.6	1.1	1.7	1.7	3.4	3.6	2.8	2.7	3.3	2.8	b
2.2	2.2	1.5	1.7	5.1	6.0	3.0	3.0	4.3	4.0	a
8.8	2.2	1.0	1.5	3.7	6.7	3.2	3.6	4.5	3.9	a
17.6	2.2	1.0	1.0	3.1	3.7	2.7	2.4	3.9	3.1	b
Mean		1.8 c[2]	1.6 c	4.5 a	4.3 a	2.9 b	3.2 b	4.2 a		

[1]Significance: Hybrids (H) ($P \leq 0.001$); Grow regulators (GR) ($P \leq 0.001$); HxGR ($P \leq 0.05$)

[2]Different letters indicate significant differences according to Duncan's test ($P = 0.05$).

Table 4. Effect of BA and NAA on average fresh weight (mg) from segments of shoot clusters in hybrid lilies after 6 weeks in culture on MS medium.

Growth Regulators (μM)		Hybrid lines							Mean
		12	00151	00150	00144	409	599	20	
BA	NAA								
2.2	1.1	722	237	1686	780	548	467	1888	1163 b[2]
8.8	1.1	415	250	2401	2590	482	929	2128	999 b
17.6	1.1	594	239	974	1052	609	1897	1371	976 b
2.2	2.2	585	255	1800	845	534	1091	2311	1377 b
8.8	2.2	750	260	809	1630	841	1967	3072	2409 a
17.6	2.2	360	253	1050	935	623	333	1981	1385 b
Mean		477 d[2]	248 d	1513 b	1347 b	600 cd	1102 bc	2233 a	

[1]Significance: Hybrids (H) (P≤0.001); Grow regulators (GR) (P≤0.05); HxGR (P≤0.05)

[2]Different letters indicate significant differences according to Duncan's test (P = 0.05).

Table 5. Effect of BA and NAA on root number formed from segments of shoot clusters in hybrid lilies after 6 weeks in culture on MS medium[1].

Growth Regulators (μM)		Hybrid lines							Mean	
		12	00151	00150	00144	409	599	20		
BA	NAA									
2.2	1.1	2.3	0.3	4.6	5.2	4.2	5.2	5.2	3.9	ab[2]
8.8	1.1	1.0	0.3	2.5	2.3	3.1	2.5	2.9	2.1	cd
17.6	1.1	1.0	0.2	1.5	1.2	1.5	1.1	1.2	1.1	d
2.2	2.2	3.1	0.2	5.8	6.7	4.7	6.0	6.5	4.7	a
8.8	2.2	2.0	0.2	3.8	4.2	1.7	3.2	4.6	2.8	ab
17.6	2.2	0.7	0.2	1.0	2.5	0.9	2.1	1.8	1.3	cd
Mean		1.7 bc[2]	0.2 c	3.2 a	3.7 a	2.7 b	3.4 a	3.7 a		

[1]Significance: Hybrids (H) (P≤0.05); Grow regulators (GR) (P≤0.01); HxGR (NS)

[2]Different letters indicate significant differences according to Duncan's test (P = 0.05).

Figures

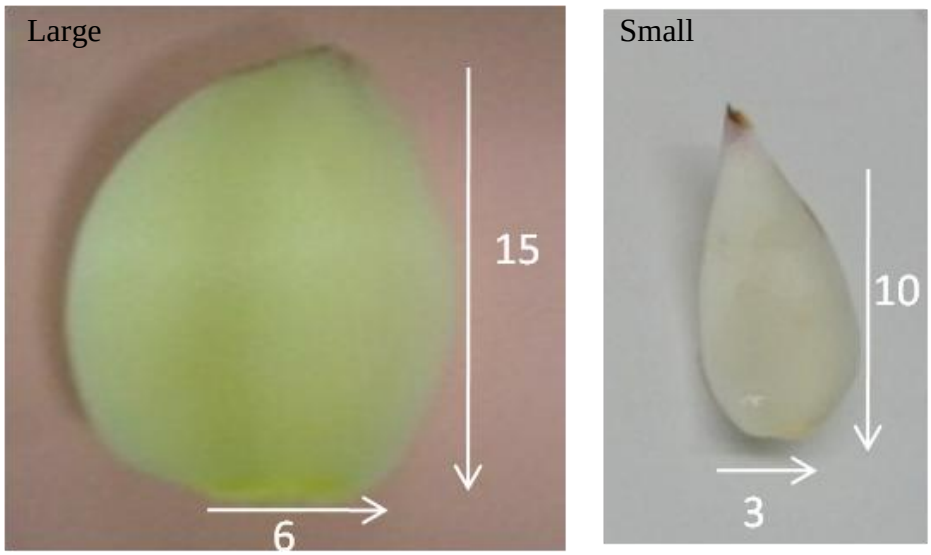


Figure (not in Acta Hort.). Bulb scales segments (large and and small) of Asiatic hybrid lilies.

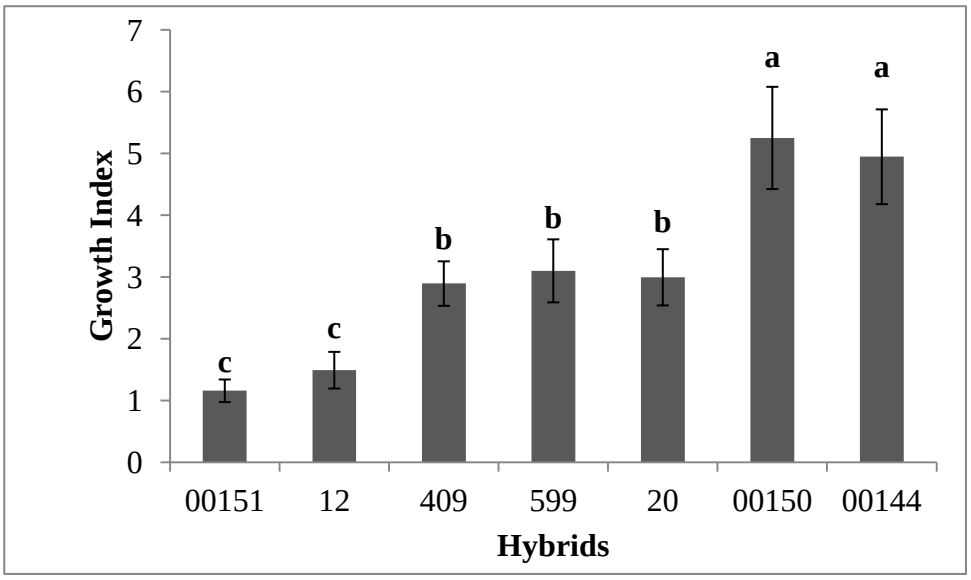


Fig. 1. Growth index between subcultures two and three in hybrid lilies. Different letters indicate significant differences according to Duncan's test (P = 0.05).

2.2 Micropropagazione del lilium in bioreattore: vantaggi e criticità del sistema (**Cardona Suárez C.M.,** Colla G., Cardarelli M. (2012). Acta Italus Hortus 6: 119-121)

2.2.1 ABSTRACT

Scaling-up in bioreactors and reduction in manual handling provide an efficient and economic system for in vitro multiplication of bulb plants. In vitro bulblet propagation of Lilium was studied using an automated balloon type bubble bioreactor. Two different culture methods were compared: continuous immersion and temporary immersion in liquid medium (ebb and flood system). Results revealed that morphological traits and biomass accumulation were more efficient when culturing was performed under continuous immersion. It was found a technique to avoid contamination of the substrate during the cultivation in the bioreactor. This technique includes the sterilization of Lilium clusters with sodium hypochlorite for 5-15 minutes before setting up the bioreactor and the use of cefotaxime in the liquid medium.

Key words: Liquid culture, ebb and flood, immersion culture.

Introduzione

L'utilizzo in vitro dei bioreattori, poichè prevede l'impiego del mezzo liquido come substrato, oltre a favorire lo scambio di nutrienti fra tessuto vegetale e mezzo di coltura, può agevolare l'automazione delle operazioni manuali e consentire una significativa riduzione dei costi della micropropagazione. Tale tecnologia potrebbe risultare particolarmente vantaggiosa per il Lilium il quale è già efficacemente moltiplicato in vitro a partire da scaglie per una produzione massale (Han 2004). Obiettivo della ricerca è stato quello di valutare l'adattabilità del bioreattore e del metodo di coltura (immersione temporanea o immersione continua) alla moltiplicazione del Lilium evidenziando potenzialità e difficoltà del sistema.

Materiale e metodi

L'esperimento è stato condotto ponendo all'interno di bioreattori (Balloon Type Bubble Bioreactor) clusters (5-7 mm) di germogli dell'ibrido commerciale (LA) Brindisi® sviluppatisi alla base di scaglie incubate per 5 settimane su substrato solido (Cardona Suárez et al., 2010). Per ogni bioreattore (volume 4 litri) è stato utilizzato 1 litro di

substrato contenente 4,4 g l-1 di sali MS (Murashige e Skoog, 1962), 4,4 µM BA, 1,1 µM NAA, 6% saccarosio (pH 5,8). Sono stati utilizzati 20-30 germogli, corrispondenti a circa 15 g di materiale vegetale, per bioreattore. All'interno dei bioreattori è stata inserita una rete per il sostegno del materiale vegetale. Le condizioni ambientali prevedevano una temperatura di 24±1°C e buio continuo. Il primo esperimento è stato finalizzato ad individuare una tecnica in grado di evitare possibili contaminazioni a carico del substrato durante la coltura in bioreattore. Per questo, il materiale vegetale (già proveniente da vitro) è stato ulteriormente sottoposto a sterilizzazione con ipoclorito di sodio (2% di cloro attivo) seguito da 3 lavaggi con acqua sterile prima dell'allestimento del bioreattore. I trattamenti previsti erano: ipoclorito per 15 minuti; ipoclorito per 15 minuti e poi aggiunta di cefotaxime 0,50 mg ml-1 nel substrato liquido; ipoclorito per 5 minuti e poi cefotaxime 0,50 mg ml-1 nel substrato liquido. Il controllo prevedeva l'allestimento di bioreattori senza alcun trattamento preventivo con ipoclorito e/o antibiotico. I bioreattori sono stati impostati secondo il sistema ad immersione temporanea (TIS) con 30 minuti di immersione ogni 2 ore e mezza per un totale di 8 cicli/giorno. Nel corso del secondo esperimento il sistema ad immersione continua (con 0,1= volume d'aria/ volume totale, minuto) è stato messo a confronto con il sistema TIS; contemporaneamente è stata avviata una prova su substrato solido in barattoli di vetro contenenti 200 ml dello stesso substrato di cui sopra aggiunto di agar allo 0,7%. Anche in questo caso il materiale vegetale è stato preventivamente sterilizzato con ipoclorito per 15 minuti e poi è stato aggiunto cefotaxime 0,50 mg ml-1 nel substrato liquido. In entrambi gli esperimenti, per ciascun trattamento sono state effettuate 3 repliche. Dopo 4 settimane sono stati rilevati i dati biometrici mentre una parte dei bulbi è stata utilizzata per determinare il peso fresco ed il peso secco (dopo aver essiccato i bulbi ad 80°C per 48 ore). Quindi sono stati calcolati l'indice di crescita (Morán et al., 2003) e la percentuale di sostanza secca. I dati sono stati sottoposti all'analisi della varianza, previa trasformazione angolare dei valori percentuali, e le medie delle variabili risultate significative sono state confrontate con il test di Duncan ($p = 0,05$).

Risultati e discussione

Tutti i trattamenti con ipoclorito e/o con antibiotico hanno determinato una totale inibizione della comparsa di inquinamenti batterici mentre nel controllo, ossia in assenza di trattamenti, già dopo 5-7 giorni il substrato all'interno dei bioreattori appariva irrimediabilmente contaminato impedendo il proseguimento della coltura di

germogli. Rispetto al trattamento di sterilizzazione, l'immersione del materiale vegetale con ipoclorito alla più bassa concentrazione e la presenza di antibiotico nel substrato hanno indotto un incremento significativo del numero di germogli prodotti dopo appena 4 settimane da ogni singolo espianto (Tab. 1); anche l'altezza e il diametro dei germogli ne sono risultati influenzati positivamente. Per quanto riguarda il peso fresco (sia totale che di ogni espianto) e la formazione di radici tali parametri hanno mostrato valori superiori in presenza di antibiotico così come osservato da precedenti autori (Kaur et al., 2008; Luna et al., 2009). Per quanto riguarda il metodo di coltura, l'utilizzo di bioreattori ha permesso un incremento della maggior parte dei parametri biometrici osservati rispetto alla propagazione su substrato solido. In particolare l'immersione continua è risultata migliore rispetto al sistema TIS e a sua volta il sistema TIS è risultato più idoneo rispetto al substrato solido per i seguenti parametri: altezza dei germogli, peso fresco totale della massa vegetale prodotta, peso fresco di ogni singolo espianto, indice di crescita. In particolare l'indice di crescita calcolato nel caso dell'immersione continua era almeno 3 volte superiore rispetto al valore riscontrato su substrato solido mentre il peso fresco totale ha evidenziato un aumento superiore di 10 volte rispetto ai germogli in coltura con agar. Il numero di germogli ottenuti dopo 4 settimane da ogni espianto, anche se non significativamente diverso rispetto al tipo di trattamento, è risultato più elevato in bioreattore rispetto al substrato solido e, soprattutto, nel caso del metodo ad immersione continua. Nessuna differenza è stata riscontrata fra i trattamenti per la percentuale di sostanza secca né per la presenza di malformazioni ed inoltre non sono stati osservati fenomeni di vitrificazione (dati non mostrati) a carico dei tessuti vegetali (Lian et al., 2003a). La radicazione è risultata stimolata nel caso del trattamento in bireattore con immersione temporanea (Tab. 2). I risultati trovano conferma con quanto riportato da Lian et al. (2003b) secondo i quali la moltiplicazione del Lilium in bioreattore ad immersione continua permette una più elevata produzione di biomassa vegetale rispetto al sistema TIS poiché nel primo caso i tessuti restano continuamente a contatto con il substrato liquido con un migliore assorbimento dei nutrienti. Risultati analoghi sono stati evidenziati anche per altre specie vegetali quali Spathiphyllum cannifolium (Dewir et al., 2006), Alocasia amazonica (Jo et al., 2008), Allium sativum (Kim et al., 2004), crisantemo (Hahn e Paek, 2005), Oncidium (Yang et al., 2010). Nel caso dell'immersione continua, è interessante evidenziare che si tratta di un sistema di più facile realizzazione rispetto al sistema TIS in quanto non sono necessari temporizzatori e valvole solenoidi per la programmazione del flusso e riflusso; tutto questo fa ipotizzare una più facile gestione

del sistema in ambito aziendale con minori criticità di funzionamento (Lian et al., 2003b). I germogli ottenuti sono stati ambientati con una percentuale di sopravvivenza del 100% per entrambi i trattamenti in bioreattore mentre nel caso del substrato solido tale percentuale si è ridotta al 75%. Tale risultato è da ricondurre alle dimensioni dei germogli che, se ottenuti in bioreattore, arrivavano ad un peso di circa 4-5 g ossia ad un valore doppio di quello misurato su substrato solido, garantendo una emergenza ottimale in vivo (Lian et al., 2003b).

Riassunto

Il Lilium è stato vantaggiosamente moltiplicato in vitro in bioreattore (Balloon Type Bubble Bioreactor) con risultati ottimali con il metodo ad immersione continua rispetto al sistema a immersione temporanea per i seguenti parametri: altezza dei germogli, peso fresco totale della massa vegetale prodotta, peso fresco di ogni singolo espianto, indice di crescita. E' stata inoltre individuata una tecnica in grado di evitare possibili contaminazioni a carico del substrato durante la coltura in bioreattore; tale tecnica prevede la sterilizzazione del materiale vegetale con ipoclorito di sodio (2% di p.a.) per 5-15 minuti prima dell'allestimento del bioreattore e l'utilizzo di cefotaxime 0,50 mg ml-1 nel substrato liquido di coltura.

Bibliografia

CARDONA SUÁREZ C.M., COLLA G., CARDARELLI M., 2011. Response to in vitro propagation of different Asiatic hybrids lilies. Acta Hortic. 900: 393-400.

DEWIR Y.H., CHAKRABARTY D., HAHN E.J., PAEK K.Y., 2006. A simple method for mass propagation of Spathiphyllum cannifolium using an airlift bioreactor. In Vitro Cell. Dev. Biol.- Plant 42: 291-297.

HAN B.H., YU H.J., YAE B.W., PEAK K.Y., 2004. In vitro micropropagation of Lilium longiflorum 'Georgia' by shoot formation as influenced by addition of liquid medium. Sci. Hortic. 103: 39-49.

HAHN E.J., PAEK K.Y., 2005. Multiplication of Chrysanthemum shoots in bioreactors as affected by culture method and inoculation density of single node stems. Plant Cell Tiss. Org. Cult. 81: 301-306.

JO U.A., MURTHY H.N., HAHN E.J., PAEK K.Y., 2008. Micropropagation of Alocasia amazonica using semisolid and liquid cultures. In Vitro Cell. Dev. Biol.-Plant 44: 26-32.

KAUR A., GILL M.S., RUMA D., GOSAL S.S., 2008. Enhanced in vitro shoot multiplication and elongation in sugarcane using cefotaxime. Sugar Tech 10 (1): 60-64.

KIM E.K., HAHN E.J., MURTHY H.N., PAEK K.Y., 2004. Enhanced shoot and bulblet proliferation of garlic (Allium sativum L.) in bioreactor systems. J. of Hort. Sci. and Biotech., 79 (5): 818-822.

LIAN M.L., CHKRABARTY D., PAEK K.Y., 2003a. Bulblet formation from bulbscale segments of lilium using bioreactor system. Biologia Plantarum 46 (2): 199-203.

LIAN M.L., CHKRABARTY D., PAEK K.Y., 2003b. Growth of Lilium Oriental Hybrid 'Casablanca' bulblet using bioreactor culture. Scientia Hort. 97: 41-48.

LUNA C., COLLAVINO M., SANSBERRO P., MROGINSKI L., 2009. Bacterial contamination in Ilex dumosa (aquifoliaceae) cultures: Antibiotic treatment. Acta Hort. 812: 97-102.

MORÁN G.P., COLQUE R., VILADOMAT F., BASTIDA J., CODINA C., 2003. Mass propagation of Cyrtanthus clavatus and Cyrtanthus spiralis using liquid medium culture. Sci. Hort. 98: 49-60.

MURASHIGE T., SKOOG F., 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Phisiol. Plant. 15: 473-497.

YANG J.F., PIANO X.C., SUN D., LIAN M.L., 2010. Production of protocorm-like bodies with bioreactor and regeneration in vitro of Oncidium 'Sugar Sweet'. Sci. Hort. 125: 712-717.

Tabella 1 – Effetto di trattamenti per la contaminazione batterica sui parametri morfologici di germogli di *Lilium* ottenuti dopo quattro settimane in bioreattore ad immersione temporanea.
Table 1 – Effect of treatments for bacterial contamination on morphological traits of Lilium shoots obtained after four weeks in a temporary immersion bioreactor.

Rilievi	Trattamento		
	Ipoclorito 15 min	Ipoclorito 15 min + cefotaxime	Ipoclorito 5 min + cefotaxime
Germogli/espianto (n.)	1,9 b	2,0 b	2,7 a
Altezza germogli (cm)	8,5 b	9,5 b	11,31 a
Diametro germogli (cm)	0,9 c	1,3 b	1,5 a
Peso fresco totale (g)	60,1 b	102,7 a	113,8 a
Peso fresco espianto (g)	2,2 b	3,9 a	4,4 a
Indice di crescita	5,0 b	9,3 a	10,4 a
Sostanza secca (%)	9,4 b	11,1 a	10,5 ab
Malformazioni (%)	2,1 n.s.	2,5 n.s.	1,7 n.s.
Radici/espianto (n.)	1,1 b	5,3 a	5,1 a

Lettere diverse sulla riga indicano una differenza significativa per p = 0,05 (test di Duncan).

Tabella 2 – Effetto del metodo di coltura sui parametri morfologici di germogli di *Lilium* ottenuti dopo quattro settimane in bioreattore.
Table 2 – Effect of culture method on morphological traits of Lilium shoots obtained after four weeks in bioreactor.

Rilievi	Substrato liquido		Substrato solido
	Immersione continua	Immersione temporanea	
Germogli/espianto (n.)	2,5 a	2,0 ab	1,8 b
Altezza germogli (cm)	15,0 a	9,5 b	4,9 c
Diametro germogli (cm)	1,0 b	1,3 a	0,9 b
Peso fresco totale (g)	131,1 a	102,7 b	9,2 c
Peso fresco espianto (g)	5,1 a	3,9 b	1,0 c
Indice di crescita	12,0 a	9,3 b	3,6 c
Sostanza secca (%)	12,3 n.s.	11,1 n.s.	11,9 n.s.
Malformazioni (%)	1,3 n.s.	2,5 n.s.	3,7 n.s.
Radici/espianto (n.)	1,8 b	5,3 a	1,7 b

Lettere diverse sulla riga indicano una differenza significativa per p = 0,05 (test di Duncan).

Figures

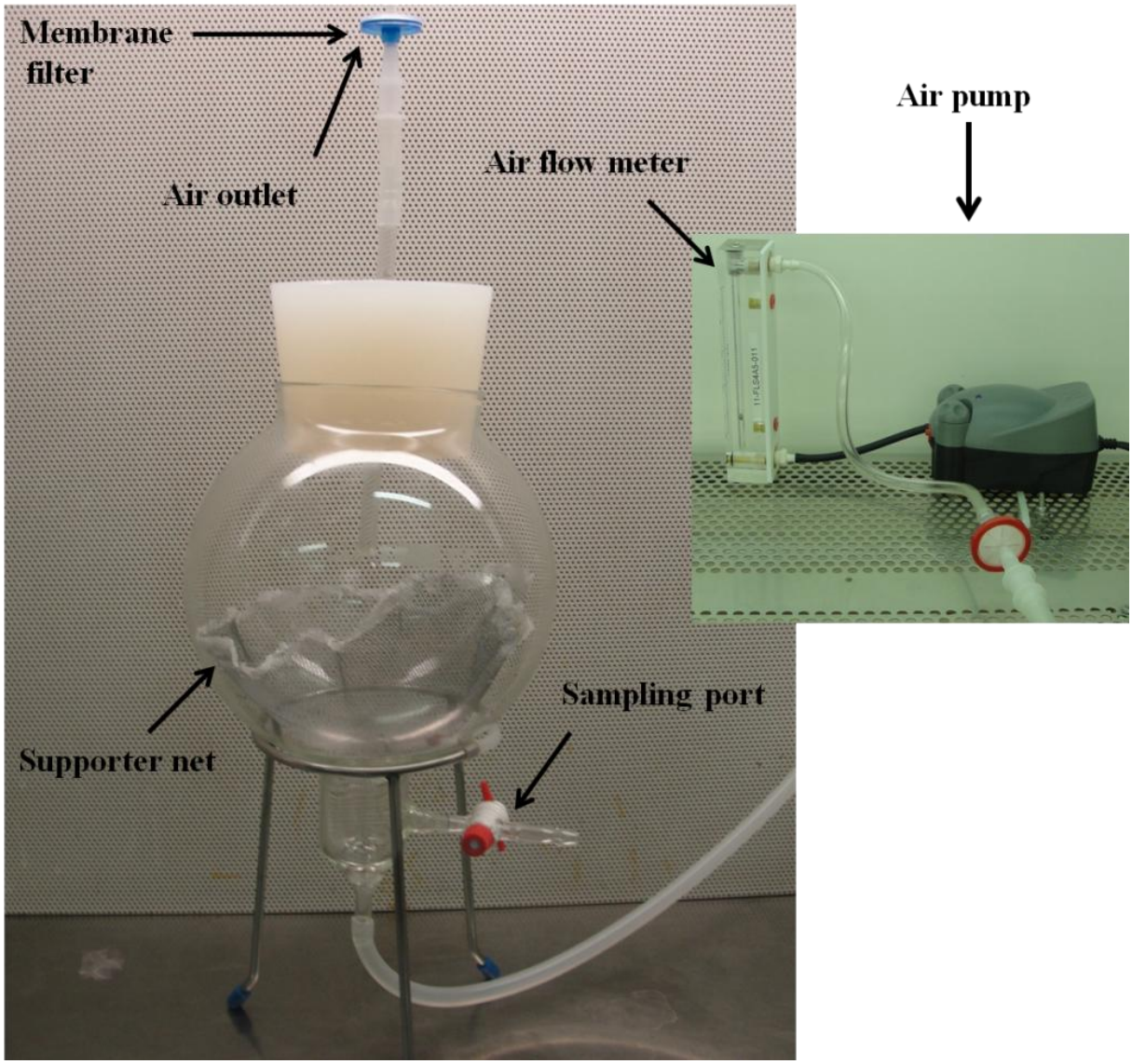


Figure (not in Acta Italus Hortus). Schematic diagram of Balloon-type bubble bioreactor (BTBB).

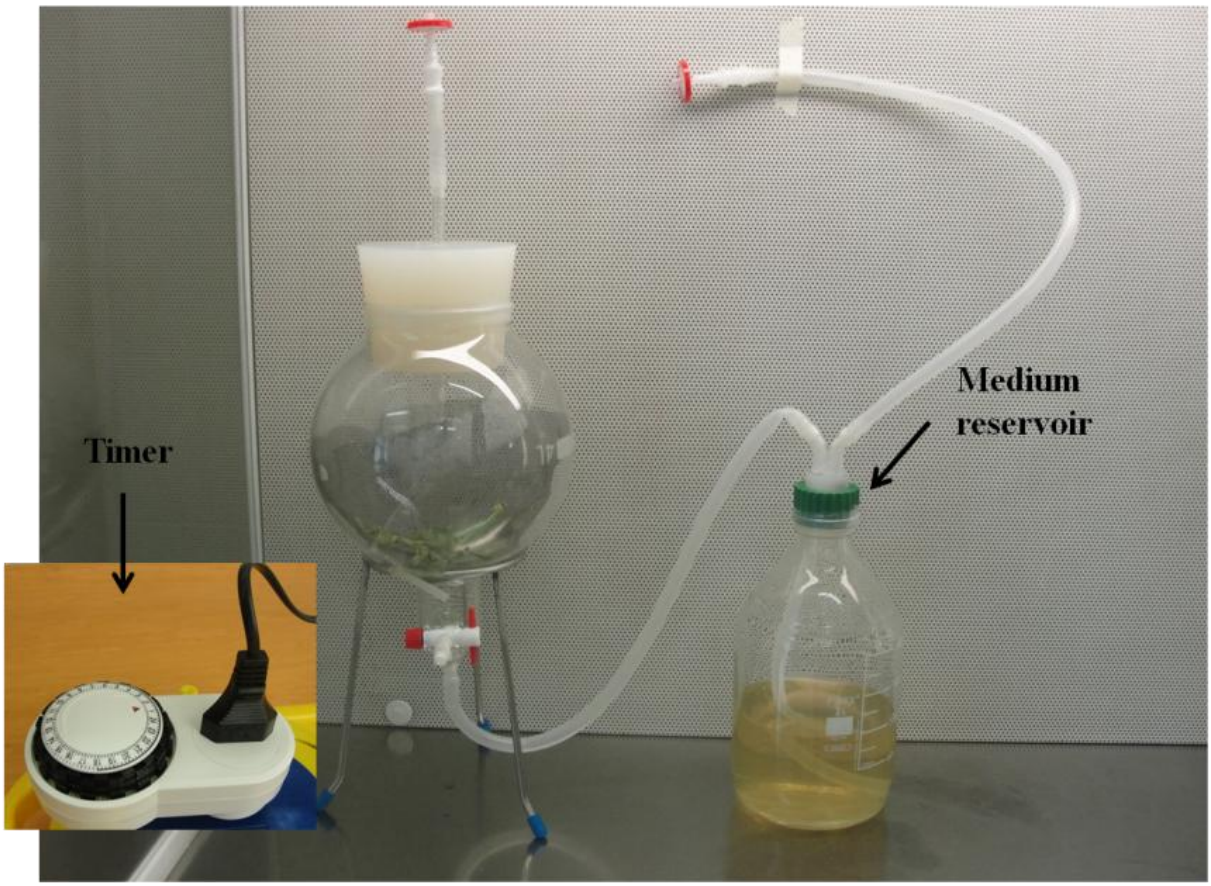


Figure (not in Acta Italus Hortus). Schematic diagram of TIS -Temporary immersion System (ebb & flood) in Balloon Type Bubble Bioreactor.

Figure (not in Acta Italus Hortus). Contaminated bulblets of *Lilium* LA. Hybrid "Brindisi" after 7 days of bioreactor culture without sterilization treatments

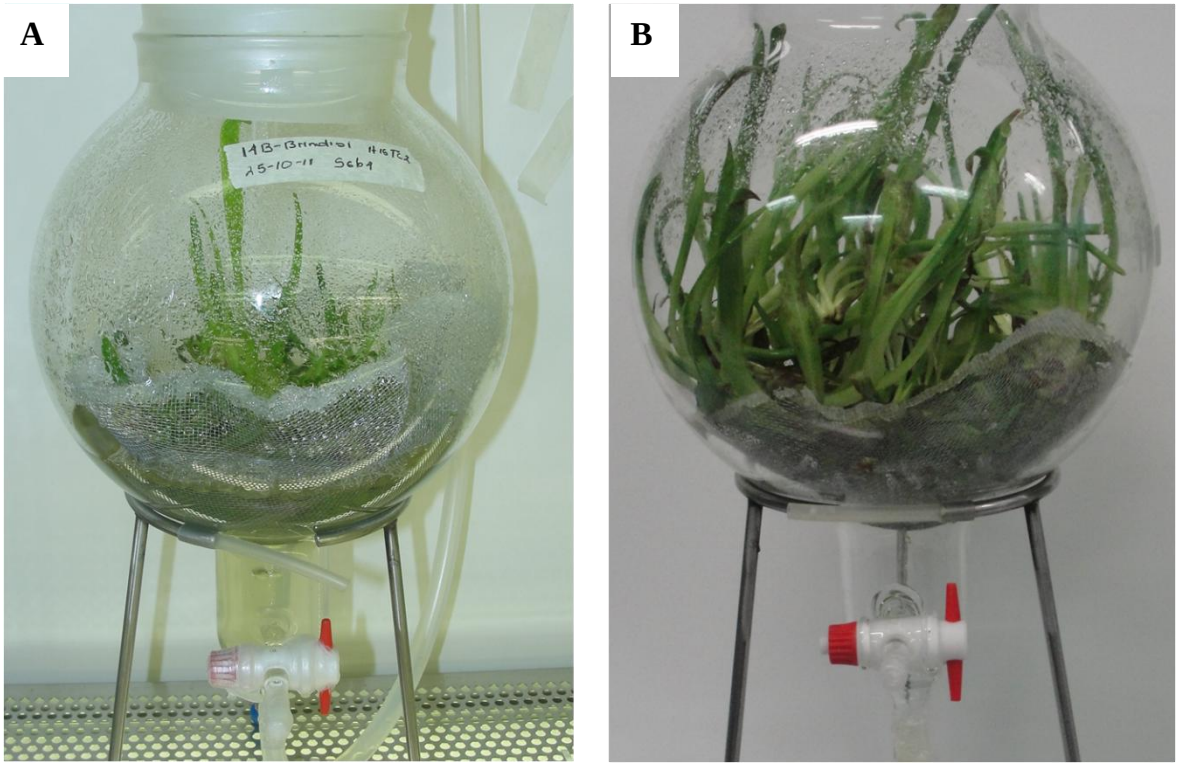


Figure (not in Acta Italus Hortus). 4: Bulblet growth of *Lilium* (LA) hybrid Brindisi as affect by different treatments for bacterial contamination after 4 weeks in a temporary immersion bioreactor. (**A)** Hypochorite 15 min. (**B)** Hypochorite + Cefotaxime.

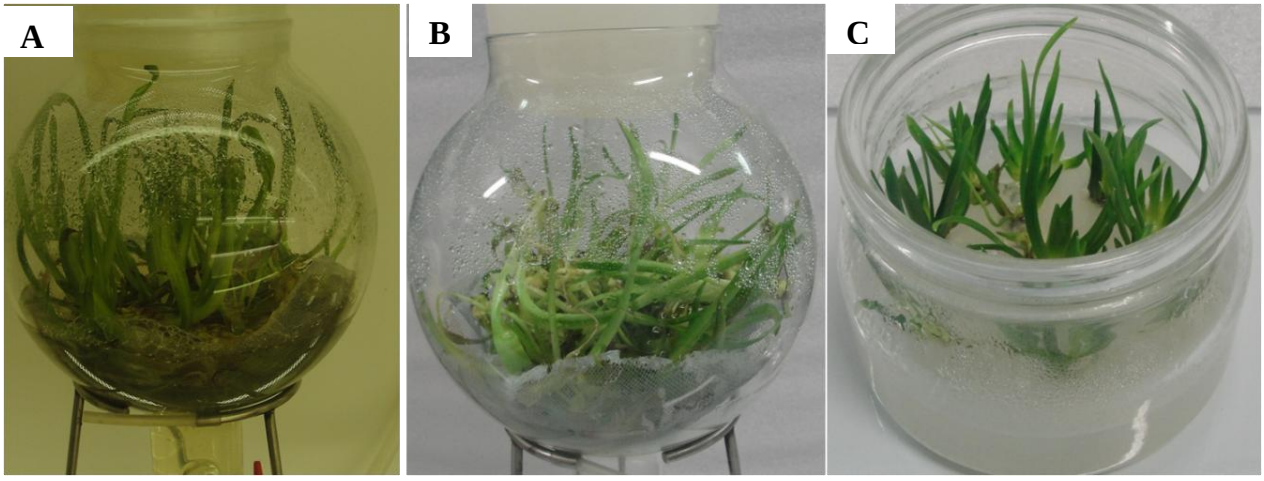


Figure (not in Acta Italus Hortus). Bulblet growth of *Lilium* (LA) hybrid Brindisi in **(A)** continuos immersion bioreactor, **(B)** temporary immersion bioreactor and **(C)** solid medium after 4 weeks of culture.

2.3 INFLUENCE OF OZONE TREATMENTS ON *IN VITRO* PROPAGATION OF *Aloe barbadensis* IN CONTINUOUS IMMERSION BIOREACTOR

2.3.1 ABSTRACT

A feasible methodology for commercial production from shoots of *Aloe barbadensis* Mill. (Liliaceae) was developed in a continuous immersion bioreactor. The values of fresh weight (total and of shoot clusters), growth index, shoots per explants, shoot length and diameter were higher in bioreactor system than on solid substrate. A better quality of shoots obtained in bioreactor was confirmed by FRAP assay, MDA and phenol contents that were reduced as compared with solid culture. O_3 treatments (zero min, 1 min, 5 min or 15 min per day) were performed to avoid the risk of contamination in bioreactor and no visible symptoms of hyperhydricity were detected with or without O_3. Highest values of total fresh weight, growth index and fresh weight of shoot clusters were observed in the control and with 15 min of O_3. Levels of APX, FRAP assay, MDA and phenol content were upper in shoot grown under O_3 exposure as a direct sign of oxidative stress. O_3 was successfully applied during the micropropagation in bioreactor showing firstly some data on the O_3 sensivity of *in vitro* plants. Higher concentration of sucrose (60 g l^{-1}) induces a better shoot proliferation and dry matter of explants than with 30 g l^{-1} of sucrose. Also APX, MDA and FRAP assay and total phenolic contents were significantly higher with 60 g l^{-1} of sucrose. Results provide experimental evidence that the application of continuous immersion bioreactors for the micropropagation of *A. barbadensis* associated with O_3 treatments is a viable and efficient model for automation and large-scale production of quality shoots.

Keywords BTBB type - liquid medium - ozone sterilization - antioxidant enzymes - sucrose concentration

Abbreviations

BA 6-benzyladenine

IAA indole-3-acetic acid

BTBB balloon type bubble bioreactor

FRAP ferric reducing-antioxidant power

PVPP polyvinilpolypirrolidone

APX ascorbate peroxidase

CAT catalase

TBARS thiobarbituric acid-reactive substances

TBA thiobarbituric acid

TCA trichloroacetic acid

MDA malonyldialdehyde

ROS toxic reactive oxygen species

2.3.2 INTRODUCTION

Aloe barbadensis Mill. is an important medicinal plant belonging to the Asphodelaceae family. This species has been used for centuries in traditional and folk medicine to treat several health disorders. Nowadays its leaves are processed by industry as source of pharmaceutical, cosmetics and healthy food products. Although *A. barbadensis* can be vegetatively propagated through lateral buds, this method is slow and labor intensive and few propagules are produced from a single stock plant (Hashem Abadi and Kaviani, 2010). Tissue culture is an alternative propagation method to produce a great number of high quality plantlets and to increase the plant growth rate allowing large-scale production. *In vitro* propagation of *A. barbadensis* through axillary shoot formation has been reported by some authors (De Oliveira et al., 2009; Hashem Abadi and Kaviani, 2010; Natali et al., 1990; Thind et al., 2008). However, all reported systems used gelled medium; so that they are costly and laborious due to the intensive manual handling required (Alper et al., 1994). Automation of micropropagation in bioreactors is a possible way of reducing costs of propagation, as reported by Adelberg and Fári (2010). Recently, mass propagation of plant tissue organs in bioreactor systems has been achieved in many plant species, including lily (Cardona Suárez et al., 2012; Ho et al., 2006; Lian et al., 2003), potato (Kämäräinen-Karppinen, 2010; Piao et al., 2003), *Stevia rebaudiana* (Sreedhar et al., 2008), raspberry (Arencibia et al., 2013), apple (Chakrabarty et al., 2007), pineapple (Escalona et al., 2003; Scherer et al., 2013), *Xanthosoma sagittifolium* (Niemenak et al., 2013), strawberry (Debnath, 2008), plantain (Aragón et al., 2010; Roels et al., 2005), chrysanthemum (Sivakumar et al., 2005), *Lessertia frutescens* (Shaik et al., 2010), *Oncidium* (Yang et al., 2010), and *Pueraria tuberosa* (Sharma et al., 2011). However, no studies in *A. barbadensis* using bioreactors have been yet published.

The aim of this study was to provide a feasible methodology for commercial production of *A. barbadensis* in a continuous immersion bioreactor. For this reason the performances of aloe shoots in bioreactor have been compared with those of solid substrate. Moreover, we investigated the effect of ozone (O_3) and of different sucrose concentrations on multiplication and growth of *A. barbadensis* shoots. The O_3 treatments were intended to reduce the high risk of contamination in bioreactors caused by the presence of bacteria within the plant tissues (Dewir et al., 2005). In fact ozone is a powerful oxidant principally applied as disinfectant of drinking and waste water (Hassenberg et al., 2007) and the hypothesis was that it could replace the conventional disinfection methods (e.g. with sodium hypochlorite and ethanol and/or antibiotics) commonly used in micropropagation. These chemical methods do not ensure an effective sterilization within the bioreactor system (Luna et al., 2009), as we have been able to verify during repeated experiments (Cardona Suárez et al., 2012).

2.3.3 MATERIALS AND METHODS

2.3.3.1 Plant material and establishment of shoot culture

Lateral shoots were excised from potted plants of *A. barbadensis* and washed with running tap water for 10 min; then they were surface-sterilized by sequential immersions in 70% (v/v) ethanol for 1 min and 2% (v/v) sodium hypochlorite for 15 min, and finally rinsed four times with sterile distilled water. Shoot basal parts, cut to have 2.0 cm long explants, were placed in glass jars containing 200 ml MS salt medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 8.8 µM BA, 1.1 µM IAA (Borgognone et al., 2013), and 0.7% (w/v) purified agar (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The medium was adjusted to pH 5.8 and autoclaved for 20 min (at 121°C/103 kPa). The cultures were maintained at 25°C±1°C with a 16-h photoperiod under white fluorescent lights (40 µmol m^{-2} s^{-1}). After 7 weeks, new developed shoots (of about 1.0 g FW) were used as explants for three subsequent experiments.

2.3.3.2 Experiment 1: Comparison between solid culture and continuous immersion bioreactor

For solid cultures, 5 shoots (approximately 5 g FW) were placed in a glass jar containing 200 ml of MS salt medium with 60 g l^{-1} sucrose, 8.8 µM BA, 1.1 µM IAA,

and 0.7% (w/v) purified agar (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Two vessels were used in each replicate. The medium was adjusted to pH 5.8 and autoclaved for 20 min (at 121°C/103 kPa).

Continuous immersion bioreactors with net (to avoid complete submersion of explants in the liquid medium) were used to select a suitable method for *in vitro* growth and multiplication on liquid substrate of *A. barbadensis*. Twenty shoots [20 g fresh weight (FW) of inoculum] were transferred to 4 liter balloon type bubble bioreactor (BTBB) with 1 liter of MS liquid medium supplemented with 60 g l^{-1} sucrose, 8.8 µM BA and 1.1 µM IAA (Fig. 1). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C/103 kPa for 20 min. The volume of input air was adjusted to 0.1 vvm (air volume culture volume^{-1} min^{-1}). All cultures (jars and bioreactors) were maintained at 25°C±1°C with a 16-h photoperiod under white fluorescent lights (40 µmol m^{-2} s^{-1}) for 4 weeks.

2.3.3.3. Experiment 2: Ozone treatments during continuous immersion bioreactor culture

Because the ultimate goal of experiment was to establish a protocol repeatable even at the enterprise level, great attention has been directed to the identification of treatments that could eliminate the risk of contaminations. Ozone was generated by corona discharge (ozone tube) using ambient air with an O_3 generator (Model OZX-300AT ENALY M&E, Shanghai, China) having a maximum ozone output of 200 mg h^{-1} at a flow rate of 1.0-2.0 liter min^{-1}. Ozone has been made to flow inside the continuous immersion bioreactors together with the air inlet. Four different treatments were daily carried out for the entire period of the trial (4 weeks): 0 min O_3 per day (control treatment); 1 min O_3 per day; 5 min O_3 per day; 15 min O_3 per day. Liquid substrate and growth conditions of bioreactors were those described in the previous experiment.

2.3.3.4. Experiment 3: Use of different sucrose concentrations in bioreactor culture

To investigate the optimal sucrose concentration in the substrate, two different concentrations (30 or 60 g l^{-1}) were tested in bioreactor with MS, 8.8 µM BA and 1.1 µM IAA (pH 5.8). Growth conditions of bioreactors were those described in the previous experiment. In this experiment was applied 15 minutes of O_3 per day of bioreactor culture.

2.3.3.5 Measures

Several growing parameters were recorded at the end of each experiment (after 4 weeks): number of shoots per explant, shoot length, shoot diameter, fresh weight of each shoot cluster, total fresh weight, number of roots per explant, root length, root fresh weight, percent of hyperhydric shoots. Dry weights were determined after drying for 48 h at 70°C. Growth index (GI) was calculated as described by Russowski et al. (2006) [GI = (weight final – weight initial) weight initial^{-1}]. A part of the plant material was immediately frozen in liquid nitrogen and maintained at -80 °C for further analysis (malondialdehyde content, enzymes assays, total antioxidant capacity by FRAP assay, total phenol content).

2.3.3.6. Antioxidant enzyme assay

Enzyme extractions were performed using a pre-chilled mortar and pestle with two volumes of an ice-cold extraction buffer (0.05 M potassium phosphate buffer, pH 7.0) containing 0.1% (w/v) ascorbic acid, 1% (w/v) PVPP, 1 mMNa_2-EDTA and 0.1% (v/v) Triton X-100. Extracts were centrifuged at 15000 × *g* for 10 min at 4 ºC and the supernatants were used for the determination of the enzymes activity and protein content by a spectrophotometer (Helios Beta, Spectrophotometer, Thermo Electron Corporation, England). The protein concentration of the enzyme extract was determined according Bradford method (1976) using bovine serum albumin as standard.

APX (EC 1.11.1.11) activity was determined in 1 ml reaction mixture containing 50 mM K-phosphate (pH 7.0), 0.1 mM ascorbate (extinction coefficient, 2.8 mM^{-1} cm^{-1}), 0.3 mM H_2O_2. The decrease in absorbance was recorded at 290 nm for 1 min (Chen and Asada, 1989) corresponding to the oxidation of ascorbic acid.

CAT (EC 1.11.1.6) activity was directly measured by the decomposition of H_2O_2 (extinction coefficient, 0.036 mM^{-1} cm^{-1}) at 240 nm for 1 min in 1 ml assay mixture containing 50 mM K-phosphate buffer (pH 7.0), 125 mM H_2O_2 and 20 µl of the crude extract (Klapheck et al., 1990).

2.3.3.7. Determination of MDA content

Lipid peroxidation was estimated as the amount of the TBARS (mainly MDA from lipid peroxidation) determined by the TBA reaction as described by Heath and Packer (1968). Fresh frozen sample was ground in a pre-chilled mortar, and homogenized in three volumes of 0.1% (w/v) TCA. The homogenate was centrifuged at 12 000 × *g* for 15 min at 4°C and 0.4 ml of supernatant was added to 0.8 ml 0.5% (w/v) TBA in 20 %

(w/v) TCA. The mixture was incubated at 95 °C for 30 min and then quickly cooled on ice. Samples were centrifuged at 10000 × *g* for 10 min at 4°C and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.3.3.8 FRAP assay

The FRAP assay was carried out according to Benzie and Strain (1996) using a spectrophotometer (Helios Beta, Thermo Electron Corporation, England). The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol iron(III) chloride solution in the ratio 10:1:1 (v/v), respectively. Thirty microliters of sample were added to 1.0 ml of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after a 30 min incubation at 37°C. The standard curve was constructed using iron (II) sulfate solution, and the results were expressed as mmol Fe (II) g^{-1} fresh weight of plant material. All of the measurements were taken in triplicate and the mean values were calculated.

2.3.3.9 Determination of total phenolics

The amount of total phenolics was determined colorimetrically as described by Folin and Ciocalteu (1965) with modifications. After appropriate dilution (one hundred microliter aliquots of ethanol extract), 200 µl of the sample was added to 1 ml of Folin–Ciocalteu reagent (10% v/v). After 4 min, 800 µl of saturated sodium carbonate solution (7.5% w/v) was added. The absorbance at 765 nm was measured after incubation at room temperature for 1 h. Gallic acid was used to calibrate the standard curve. The value of total phenolics was expressed in terms of mg gallic acid equivalents (mg GAE) per g fresh tissue.

2.3.3.10. Measurements of chlorophyll and carotenoid contents

Samples (1 g) were prepared powdering fresh shoots in a mortar and extracted with 10 ml of 80% (v/v) cold acetone. The resulting extract was centrifuged for 20 min at 4800 × *g.* The pigment determination was spectophotometrically performed, measuring light absorbance by acetone extracts of chlorophylls a and b (663 nm and 646 nm) respectively, and carotenoids (470 nm). All amounts were expressed as µg per gram of fresh weight.

2.3.3.11 Experimental design and data analysis

The results shown are the mean values of three independent experiments. Experiments were set up in a completely randomized design and four replications were used in each treatment. Data were subjected to ANOVA and Duncan's multiple range test using SPSS program (Version 16, SAS Institute Inc., Cary, NC, USA).

2.3.4 RESULTS AND DISCUSSION

2.3.4.1 Comparison between continuous immersion bioreactor and solid culture of A. barbadensis

The present comparative study between solid culture and continuous immersion bioreactor on growth of *A. barbadensis* shoots revealed that shoot proliferation and biomass accumulation were more efficient in bioreactor system (Table 1) (Fig. 1). The values of fresh weight (total and of shoot clusters) obtained in bioreactor were up to four times higher than the values measured on solid substrate (189.70 and 11.01 versus 49.70 and 2.81). Consequently the growth index, measured with respect to changes in weight, was significantly much higher in bioreactor culture (8.49) than the value achieved during the cultivation of shoots in solid culture (1.48). Bioreactor culture also resulted in greater numbers of shoots per explants, higher shoot length and shoot diameter compared with gelled culture. Higher propagation rates in bioreactors than on gelled medium were also reported in apple (Chakrabarty et al., 2003), *Chrysanthemum* (Hahn and Paek, 2005), strawberry (Debnath, 2008) and adventitious roots of *Astragalus membranaceus* (Wu et al., 2011). The better shoot performances in the immersion bioreactor culture is linked to the fact that in liquid culture the plant tissues are continuously submerged permitting an efficient nutrient and hormones uptake which improved the growth of the plantlets (Lian et al., 2003; Yan et al., 2010). Moreover, liquid culture provides far better homogenization as compared to solid media (Sajid and Pervaiz, 2008). Adelberg et al. (2010) had shown that with gelling agents the nutrients dissolved in the liquid phase are not completely available as water is bound to the gel by a matrix force; the authors compared spent gelled medium with spent liquid medium and revealed that more nutrients remain in old agar, and therefore nutrients were somehow unavailable to the plant. Also has been shown that in presence of agar, the resistance of sucrose transfer from medium to the plant is 300 × greater than that of

sucrose transfer from a shaken liquid to the plant, per unit surface area (Adelberg and Fári, 2010).

Hyperhydricity is often associated with liquid culture (Aragón et al., 2010) and was detected in some studies concerning continuous immersion bioreactor systems (Chakrabarty et al., 2007; Shaik et al., 2010). In our results, however, the produced shoots did no present symptoms of hyperhydricity (Table 1) (Fig. 1) as observed in *Lilium* (Lian et al., 2003), *Chrysanthemum* (Hahn and Paek, 2005) and *Alocasia amazonica* (Jo et al., 2008). The use of the net to support the plant mass has certainly contributed to prevent this phenomenon ensuring a constant aeration to explants and leading to good growth of plantlets (Chakrabarty et al., 2003; Dewir et al., 2005, 2006; Paek et al., 2005).

Fresh weight of roots was no different, however the number and length of roots per explant were significantly lower in bioreactor culture as compared with solid culture (Table 1).

Higher shoot proliferation and better response of other growth parameters in bioreactor system makes the use of liquid medium an efficient system of choice for *in vitro* propagation of *A. barbadensis*. The complete elimination of agar, an expensive ingredient, in the multiplication medium has helped in a substantial cost reduction (5.2-fold) (Pati et al., 2011). Without agar, there is also a reduction of hood tranfer, that represents about 60% of the cost of a tissue cultured plant (Alper et al., 1994), and a greater efficiency in transferring plantlets to ex vitro environment (Pati et al., 2011).

Continuous immersion culture for plant propagation could induce into the plant tissues various abiotic stress increasing the level of toxic ROS (Chakrabarty et al., 2006). Enhanced activity of ROS-processing enzymes, such as CAT and APX, in shoots grown in bioreactor might suggest the occurrence of oxidative stress (Mittler, 2002). Indeed, APX enzyme activity is not significantly different between treatments indicating that APX function was performed by the other enzymes (Shigeoka et al., 2002) (Table 2). The CAT activity in bioreactor culture was significantly higher (277.60) than on from solid culture (174.70) (Table 2). Chakrabarty et al. (2007) also reported high CAT activity during growth of apple root stock in continuous immersion bioreactor. According some authors scavenging enzymes as CAT participate in regulation of plant growth during *in vitro* morphogenesis as reported for *Tacitus bellus* (Mitrović et al., 2012), gladiolus (Gupta and Datta, 2004), *Albizia odoratissima* (Rajeswari and Paliwal, 2008), strawberry (Yonghua et al., 2005) and *Crocus sativus* (Vatankhah et al., 2010). Moreover, the enhanced CAT activity in bioreactor could be associated with the initial

steps in ROS scavenging (Gillespie et al., 2011; Radhakrishnan and Lee, 2013). Indeed, MDA content in shoots obtained from bioreactor culture was significantly lower (5.59) than the value of shoots on solid culture (7.48) (Table 2). MDA is a decomposition product of polyunsaturated fatty acids hydroperoxides often utilized as biomarker for lipid peroxidation. The high MDA concentration and decreased CAT activity in solid substrate suggest that in solid culture method the antioxidative enzyme was not able to scavenge the overproduction of ROS (Ali et al., 2006; Chakrabarty and Datta, 2008; Dewir et al., 2006) resulting in shoot of worse quality than those obtained from bioreactor.

During the experiment the antioxidant capacity and the phenolic and pigments contents were also measured. In bioreactor it was observed a decrease of FRAP and total phenolic compounds values respect to gelled medium (Table 2). Several studies suggested that total phenolic contributed significantly to the antioxidant capacity (López-Berenguer et al., 2009; Oueslati et al., 2010). Chlorophyll and carotenoid contents were significantly higher in shoots grown under solid culture method compared to shoots grown under bioreactor culture (Table 2). Chlorophyll a/b ratio did not show a significant difference between solid and bioreactor method. Evidently, the *in vitro* culture conditions greatly affect the secondary metabolite production so that it's important to include an evaluation of bioactive secondary metabolite production when developing or optimizing micropropagation protocols especially of highly-utilized medicinal plants as *A.barbadensis* (Amoo et al., 2012).

2.3.4.2 Effects of ozone treatments in bioreactor culture of A. barbadensis

Morphologic parameters of *A. barbadensis* shoots obtained 4 weeks of bioreactor culture in presence and absence (control) of ozone are reported in Table 3. Highest values of total fresh weight, growth index and fresh weight of shoot clusters were observed in the control even if there weren't significant differences compared to the bioreactor with 15 min of O_3 exposure; treatments with 1 and 5 min of O_3 have shown a reduction of these parameters (Table 3). Shoot length and shoot diameter were progressively reduced from the control to treatments with 15, 5 and 1 minute of exposure to O_3. Nevertheless dry matter was significantly lower in control (8.76) and higher with 5 min of O_3 exposure (11.59). No visible symptoms of hyperhydricity were detected with or without O_3 and number of shoots per explant and root formation did not exhibit differences among all treatments (Table 3). These findings indicate that ozone treatments in bioreactor culture had no significant detrimental impacts on *in vitro*

multiplication of *A. barbadensis* (Zhang et al., 2012). The small reduction measured on some morphological parameters for treatments with 1 and 5 min of O_3 was not repeated for treatment with highest O_3 concentration that instead showed the same shoot growth of the control. Since O_3 in solution spontaneously reverts to diatomic oxygen (O_2) through complex decomposition mechanisms (Beltrán et al., 2005), the oxygen concentrations into the liquid medium increases as result of O_3 treatments. This higher O_2 availability could enhance plant growth as reported by some authors (Graham et al., 2011; Sajid and Pervaiz, 2008; Thanh et al., 2006; Zhang et al., 2012). Probably in our experiment after 15 minutes of O_3 treatment the O_2 concentration in liquid medium was high enough to increase the growth and biomass accumulation of *A. barbadensis* shoots at the levels of control.

According Zhang et al. (2012) the visible injuries on leaves are often considered as indices of plant sensitivity to ozone and sometimes these symptoms may occur without a correlate decrease of plant growth. Under 5 and 15 min of O_3 treatments, the surface of some leaves showed a few symptoms as spots, and no visible O_3 damage symptoms were observed in control and bioreactors under 1 min of O_3 exposure. Paoletti (2009) reports that if O_3 exposure is long enough, species-specific chlorotic flecking, necrosis, or bronzing may gradually coalesce on the upper leaf surface.

APX activity was significantly higher in bioreactor exposed to O_3 for 15 min and lower in other bioreactors treated with O_3 (Table 4). CAT activity didn't show significant differences between all treatments. With O_3 treatments there was an increase of damage to the lipid molecule of the cell membrane, as indicated by high values of MDA. Correspondingly, levels of FRAP assay and accumulation of phenol compounds (reported as antioxidant defense system) were upper in shoot grown under O_3 exposure as a direct sign of oxidative stress. Chlorophyll contents and Chl a/b ratio did not present significant differences among the treatments (Table 4). However plants under high O_3 exposure showed a reduction of carotenoid contents. Many researches have found that photosynthetic processes are very sensitive to high O_3 (Xu et al., 2009; Zhang et al., 2010, 2012). These results indicated that ozone sterilization in bioreactor culture had no considerable impacts in pigment contents. Chl a/b ratio was not affected by O_3 also in *Cinnamomum camphora* (Feng et al., 2011) and in *Liriodendron Chinese* (Zhang et al., 2011). However many authors (Feng et al., 2011; Xu et al., 2009; Zhang

et al., 2012) found that chlorophyll and carotenoid contents significantly decreased in plants under O_3 exposure compared with plants without O_3 treatment.

O_3 is generally detrimental to plant growth and metabolism (Fagnano, 2011; Wang et al., 2012; Zhang et al., 2011). Several authors have conducted experiments to understand the O_3 effects on many plant species under controlled conditions (greenhouses or growth chambers) or in open air. For the first time O_3 was successfully applied during the micropropagation in bioreactor, and then there was not information on the O_3 sensivity of *in vitro* plants. The hypothesis was that under the particular conditions of *in vitro* growth, O_3 should not necessarily damage the plant developments for the following reasons: a) in conventional micropropagation, the carbohydrates are added to the growing medium and the plants are thus forced to act heterotrophically or photomixotrophically, then O_3 cannot induce a decrease of photosynthesis and consequently of biomass production (Wang et al., 2012; Zhang et al., 2012); b) Zhang et al. (2012) demonstrated that O_3 can also alter stomatal conductance but during the *in vitro* culture conditions there is already a poor stomata control (Hazarika, 2006; Sáez et al., 2012); c) while the O_3 effects generally refer to chronic exposure to the gas (Paoletti, 2009), in the carried out experiments the O_3 treatments were limited to some minutes per day; d) O_3 was injected into the bioreactor through the liquid medium so that there were not a direct contact with plant tissue and also ozone is unstable in aqueous solutions (Beltrán et al., 2005).

The high risk of endophytic contamination is a serious problem for the *in vitro* propagation of plants, above all when a large number of propagules are cultured together in liquid medium (Luna et al., 2013). Cases of contamination within bioreactors have also occurred during these experiments (50% of contaminated bioreactors without ozone) (Fig. 1) showing how this critical factor may restrain the use of bioreactor systems. O_3 treatments in bioreactor culture have totally eliminated the high risk of contaminations in the system (never detectable) within 30 days of culture. Respect to other known systems of chemical sterilization (with antibiotics and/or sodium hypochlorite) (Cardona et al., 2012; Luna et al., 2008), this physic technique with ozone is high efficiency, easier to manage and less expensive. Moreover, antibiotics often exert only a bacteriostatic effect, leading to selection of resistant strains and inducing phytotoxic effects in plant tissues (Falkiner, 1998; Leifert et al., 1991). Thus O_3 treatments during micropropagation of *A. barbadensis* shoots can be

considered a useful technology to reducing or eliminate the pathogen level into bioreactors.

2.3.4.3 Effect of sucrose concentration on in bioreactor culture of A. barbadensis

Higher concentration of sucrose induces a better shoot proliferation of explants (2.51) than with 30 g l^{-1} of sucrose, as reported for *Ruta gravoelens* (Mohamed and Ibrahim, 2011) (Table 5). Also dry matter was significantly increased on medium containing more sucrose. Similar responses of increased dry matter with elevated concentrations of sucrose were reported for *Lilium* (Lian et al., 2003), *Hemerocallis* (Adelberg et al., 2010) and *Alocasia amazonica* (Jo et al., 2009). Fresh weight of roots was lowest with 60 g l^{-1} of sucrose (Table 5). No differences were observed respect to other morphological parameters. Although high sucrose concentrations result in a reduction of roots growth due to the relatively higher osmotic potential of cultured shoots (Cui et al., 2010; Jo et al., 2009), in our experiment this inhibition was not observed because on liquid medium (in bioreactor) sucrose is more available to the plants than on gelled medium (Adelberg et al., 2010). It is know that sucrose is not only an important carbon and energy source for plant cells, but it also can affects the cell metabolism and the production of metabolites (Gertlowski and Petersen, 1993). The values of APX, MDA, and antioxidant capacity by FRAP assay were significantly higher on medium supplemented with 60 g l^{-1} of sucrose as compared with medium with 30 g l^{-1} of sugar (Table 6), probably due to the oxidative stress in plant tissues (Baque et al., 2012; Cui et al., 2010; Jo et al., 2009). Several authors have highlighted that higher sucrose content in the culture medium generates osmotic stress on the tissue and can even induce an accumulation of phenol (Cui et al., 2010; Kikowska et al., 2012; Shohael et al., 2006; Wu et al., 2006). Total phenolic content was much higher for medium with 60 g l^{-1} of sucrose (511.11 µg GAE g^{-1} FW) then with 30 g l^{-1} of sucrose (351.01 µg GAE g^{-1} FW) (Table 6). Chlorophyll and carotenoid contents were the same for both sucrose concentration (Table 6). Similar response were observed by Dewir et al. (2006) during *in vitro* mass propagation of *Spathiphyllum cannifolium*.

This study provides experimental evidence that the application of continuous immersion bioreactors for the micropropagation of *A. barbadensis* is a viable and efficient model for automation and large-scale production of quality shoots. The use of O_3 inside the bioreactor is a suitable instrument to eliminate the high risk of contamination in the liquid substrate without the use of sophisticated technology. This technology could be

applied for rapid multiplication of aloe but also to produce high amount of biomass for extraction of medicinal metabolites.

Tables

Table 1 Effect of culture method (in vessel on solid substrate or in continuous immersion bioreactor) on growth of *A. barbadensis* shoots.

Parameters	Culture method		
	Solid medium	Bioreactor	
Total fresh weight (g)	49.70 ± 2.70b	189.70 ± 5.95a	***
Growth index	1.48 ± 0.09b	8.49 ± 0.30a	***
Shoots explant^{-1} (n)	1.32 ± 0.15b	2.31 ± 0.24a	***
Fresh weight of shoot cluster (g)	2.81 ± 0.17b	11.01 ± 1.32a	***
Shoot length (cm)	3.98 ± 0.15b	5.93 ± 1.13a	***
Shoot diameter (cm)	0.55 ± 0.01b	0.82 ± 0.02a	***
Dry matter (%)	10.09 ± 0.02	9.25 ± 0.94	NS
Hyperhydricity (%)	0.00 ± 0.00	0.06 ± 0.00	NS
Roots explant^{-1} (n)	3.25 ± 0.58a	1.93 ± 0.04b	*
Maximum length of roots (cm)	3.20 ± 0.61a	1.97 ± 0.11b	*
Root fresh weight (g)	0.08 ± 0.01	0.11 ± 0.04	NS

Means separation by Duncan's multiple range test, $P \leq 0.05$

NS, *, *** Non Significant or significant at $P \leq 0.05$ and 0.001, respectively

Data represent mean values (n = 4) with standard error.

Table 2 Antioxidant enzyme activities, malonyldialdehyde, antioxidant capacity, total phenolic contents and chlorophyll pigments on *A. barbadensis* shoots obtained after 4 weeks in vessel on solid substrate or in continuous immersion bioreactor.

Parameters	Culture method		
	Solid medium	Bioreactor	
Ascorbate peroxidase (mmol min^{-1} mg^{-1} protein)	6.91 ± 0.04	7.84 ± 1.63	NS
Catalase (mmol min^{-1} mg^{-1} protein)	174.70 ± 27.30b	277.60 ± 29.70a	*
Malonyldialdehyde (nmol g^{-1} FW)	7.48± 0.64a	5.59 ± 0.59b	*
FRAP assay (mmol Fe^{2+} g^{-1} FW)	17.80± 0.18a	7.88 ± 0.21b	***
Total phenolic contents (µg GAE g^{-1} FW)	390.00± 22.71a	347.30 ± 7.70b	*
Chlorophyll a (µg g^{-1} FW)	139.90± 13.20a	93.80 ± 9.76b	**
Chlorophyll b (µg g^{-1} FW)	73.40± 7.45a	46.20 ± 6.26b	**
Chlorophyll tot (µg g^{-1} FW)	213.30± 22.50a	140.00 ± 12.60b	**
Chlorophyll a/b	1.90± 0.05	2.11 ± 0.17	NS
Carotenoids (µg g^{-1} FW)	51.70± 6.07a	31.60 ± 1.18b	***

Means separation by Duncan's multiple range test, $P \leq 0.05$

NS, *, **, *** Non Significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively

Data represent mean values (n = 4) with standard error.

Table 3 Effect of ozone treatments on growth of A. *barbadensis* shoots after 4 weeks in continuous immersion bioreactor.

Parameters	Control[1]	O_3-1	O_3-5	O_3-15	
Total fresh weight (g)	174.40 ± 16.30a	140.10 ± 5.40b	135.10 ± 2.68b	158.90 ± 6.06a	*
Growth index	7.72 ± 0.82a	6.01 ± 0.27b	5.75 ± 0.13b	6.95 ± 0.03a	*
Shoots explant^{-1} (n)	2.01 ± 0.23	2.00 ± 0.08	2.05 ± 0.20	2.59 ± 0.62	NS
Fresh weight of shoot cluster (g)	8.88 ± 1.58a	6.47 ± 0.68b	6.53 ± 0.54b	7.92 ± 1.15a	**
Shoot length (cm)	5.78 ± 0.19a	4.26 ± 0.14b	4.43 ± 0.41b	4.97 ± 0.32ab	**
Shoot diameter (cm)	0.77 ± 0.01a	0.65 ± 0.04b	0.63 ± 0.04b	0.73 ± 0.01ab	***
Dry matter (%)	8.76 ± 1.12b	10.84 ± 0.17ab	11.59 ± 1.08a	10.94 ± 0.15ab	*
Hyperhydricity (%)	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	NS
Roots explant^{-1} (n)	1.90 ± 0.10	2.78 ± 0.66	2.44 ± 0.13	2.71 ± 0.40	NS
Maximum length of roots (cm)	1.85 ± 0.23	1.42 ± 0.65	1.31 ± 0.01	1.91 ± 0.12	NS
Root fresh weight (g)	0.10 ± 0.01	0.12 ± 0.02	0.11 ± 0.02	0.08 ± 0.00	NS

[1] Control: bioreactor without ozone; O_3-1: 1 min ozone per day; O_3-5: 5 min ozone per day; O_3-15: 15 min ozone per day. – Supplying ozone with a maximum ozone output of 200 mg h^{-1} at a flow rate of 1-2 liter min^{-1}

Means separation by Duncan's multiple range test, $P \leq 0.05$

NS, *, **, *** Non Significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively

Data represent mean values (n = 4) with standard error.

Table 4 Effect of ozone treatments on antioxidant enzyme activities, malonyldialdehyde, antioxidant capacity, total phenolic contents and chlorophyll pigments of *A. barbadensis* shoots obtained after 4 weeks in a continuous immersion bioreactor.

Parameters	Control[1]	O_3-1	O_3-5	O_3-15	
Ascorbate peroxidase (mmol min^{-1} mg^{-1} protein)	8.01 ± 1.20b	4.72 ± 0.45c	4.26 ± 0.56c	10.61 ± 1.63a	***
Catalase (mmol min^{-1} mg^{-1} protein)	293.10 ± 21.10	316.00 ± 36.50	234.20 ± 34.20	314.20 ± 29.90	NS
Malonyldialdehyde (nmol g^{-1} FW)	5.66 ± 0.53c	7.16 ± 0.14b	6.68 ± 0.45b	8.97 ± 0.44a	***
FRAP assay (mmol Fe^{2+} g^{-1} FW)	6.12 ± 0.45c	11.6 ± 1.39a	9.69 ± 1.84ab	8.80 ± 0.01b	**
Total phenolic contents (µg GAE g^{-1} FW)	367.00 ± 18.40b	497.11 ± 4.81a	518.51 ± 61.40a	495.29 ± 13.20a	*
Chlorophyll a (µg g^{-1} FW)	97.10 ± 7.08	99.00 ± 12.30	107.80 ± 4.12	85.90 ± 12.20	NS
Chlorophyll b (µg g^{-1} FW)	44.00 ± 6.65	42.50 ± 6.14	48.00 ± 1.20	41.50 ± 6.74	NS
Chlorophyll tot (µg g^{-1} FW)	135.30 ± 14.50	141.60 ± 18.40	155.80 ± 5.31	127.40 ± 18.90	NS
Chlorophyll a/b	2.23 ± 0.19	2.36 ± 0.04	2.27 ± 0.02	2.10 ± 0.03	NS
Carotenoids (µg g^{-1} FW)	32.4 ± 0.94ab	39.20 ± 3.29a	39.80 ± 2.37a	28.70 ± 5.51b	*

[1] Control: bioreactor without ozone; O_3-1: 1 min ozone per day; O_3-5: 5 min ozone per day; O_3-15: 15 min ozone per day. – Supplying ozone with a maximum ozone output of 200 mg h^{-1} at a flow rate of 1-2 liter min^{-1} · Means separation by Duncan's multiple range test, $P \leq 0.05$.

NS, *, **, *** Non Significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively - Data represent mean values (n = 4) with standard error.

Table 5 Effect of sucrose concentration on growth of *A. barbadensis* shoots after 4 weeks in a continuous immersion bioreactor with 15 minutes of ozone sterilization per day

Parameters	S30[1]	S60	
Total fresh weight (g)	148.10 ± 4.35	155.90 ± 6.84	NS
Growth index	6.41 ± 0.21	6.79 ± 0.34	NS
Shoots explant^{-1} (n)	1.89 ± 0.32b	2.51 ± 0.54a	*
Fresh weight of shoot cluster (g)	6.80 ± 0.84	6.98 ± 0.83	NS
Shoot length (cm)	4.98 ± 0.42	4.90 ± 0.23	NS
Shoot diameter (cm)	0.81 ± 0.06	0.72 ± 0.01	NS
Dry matter (%)	6.01 ± 0.88b	9.89 ± 0.15a	***
Hyperhydricity (%)	0.00 ± 0.00	0.01 ± 0.00	NS
Roots explant^{-1} (n)	3.04 ± 0.62	2.69 ± 0.27	NS
Maximum length of roots (cm)	1.35 ± 0.02	1.38 ± 0.05	NS
Root fresh weight (g)	0.15 ± 0.02a	0.07 ± 0.01b	*

[1] S30: 30 g l^{-1} sucrose; S60: 60 g l^{-1} sucrose

Means separation by Duncan's multiple range test, $P \leq 0.05$

NS, *, *** Non Significant or significant at $P \leq 0.05$ and 0.001, respectively

Data represent mean values (n = 4) with standard error

Table 6 Effect of sucrose concentration on antioxidant enzyme activities, malonyldialdehyde, antioxidant capacity, total phenolic contents and chlorophyll pigments of *A. barbadensis* shoots obtained after 4 weeks in a continuous immersion bioreactor with 15 minutes of ozone sterilization per day

Parameters	S30[1]	S60	
Ascorbate peroxidase (mmol min^{-1} mg^{-1} protein)	3.31 ± 0.13b	8.84 ± 0.84a	***
Catalase (mmol min^{-1} mg^{-1} protein)	282.50 ± 18.60	301.10 ± 26.20	NS
Malonyldialdehyde (nmol g^{-1} FW)	3.36 ± 0.03b	9.35 ± 0.50a	***
FRAP assay (mmol Fe^{2+} g^{-1} FW)	6.63 ± 0.11b	9.07 ± 0.01a	***
Total phenolic contents (µg GAE g^{-1} FW)	351.01 ± 9.81b	511.11 ± 8.72a	***
Chlorophyll a (µg g^{-1} FW)	84.80 ± 4.64	83.70 ± 10.30	NS
Chlorophyll b (µg g^{-1} FW)	37.80 ± 2.51	39.30 ± 4.93	NS
Chlorophyll tot (µg g^{-1} FW)	122.60 ± 4.43	125.10 ± 15.13	NS
Chlorophyll a/b	2.25 ± 0.05	2.13 ± 0.03	NS
Carotenoids (µg g^{-1} FW)	24.20 ± 2.21	26.30 ± 3.68	NS

[1] S30: 30 g l^{-1} sucrose; S60: 60 g l^{-1} sucrose

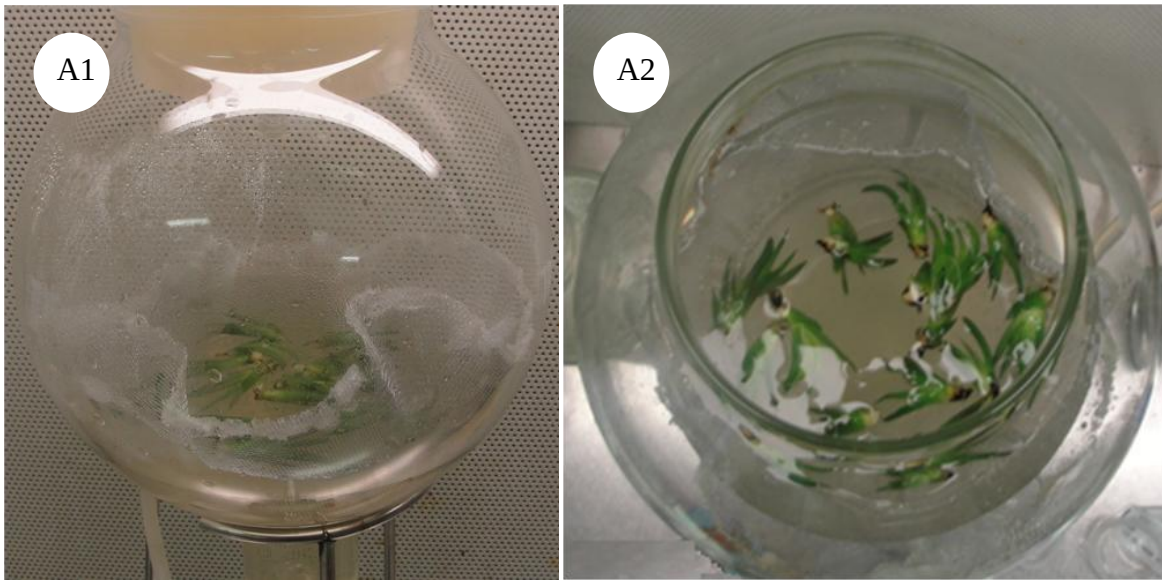
Means separation by Duncan's multiple range test, $P \leq 0.05$

NS, *** Non Significant or significant at $P \leq 0.001$, respectively

Data represent mean values (n = 4) with standard error

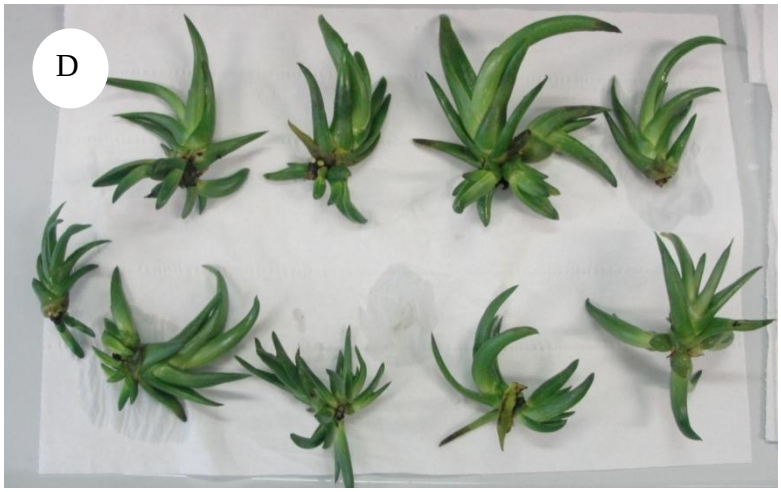
Figures

Fig. 1 *In vitro* propagation of *Aloe barbadensis* shoots: **A1, A2** initial inoculum of shoots in continuous immersion bioreactor. **B** shoots obtained in solid medium after 4 weeks of culture. **C1- C2** production of shoots in continuous immersion bioreactor after 4 weeks of culture. **D** shoots cluster of high quality grown in bioreactor.




C1


C2


D

2.3.6 REFERENCES

Adelberg, J.W., Delgado, M.P., Tomkins, J.T., 2010. Spent medium analysis for liquid culture micropropagation of *Hemerocallis* on Murashige and Skoog medium. In Vitro Cell Dev Biol - Plant 46(1), 95-107.

Adelberg, J., Fári, M.G., 2010. Applied physiology and practical bioreactors for plant propagation. Propagation Ornamental Plants 10(4), 205-219.

Ali, M.B., Hahn, E.J., Paek, K.Y., 2006. Antioxidative responses of *Echinacea angustifolia* cultured roots to different levels of CO_2 in bioreactor liquid cultures. Enzyme Microbial Technol 39(5), 982-990.

Alper, Y., Young, R.E., Adelberg, J.W., Rhodes, B.B., 1994. Mass handling of watermelon microcuttings. Trans Am Soc Agric Engineers 37(4), 1337-1343.

Amoo, S.O., Aremu, A.O., Van Staden, J., 2012. *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. Plant Cell Tissue Org Cult 111(3), 345-358.

Aragón, C.E., Escalona, M., Rodriguez, R., Cañal, M.J., Capote, I., Pina, D., González-Olmedo, J., 2010. Effect of sucrose, light, and carbon dioxide on plantain micropropagation in temporary immersion bioreactors. In Vitro Cell Dev Biol - Plant 46, 89-94.

Arencibia, A.D., Vergara, C., Quiroz, K., Carrasco, B., García-Gonzales, R., 2013. Establishment of photomixotrophic cultures for raspberry micropropagation in temporary immersion bioreactors (TIBs). Sci Hortic 160, 49-53.

Baque, M.A., Elgirban, A., Lee, E.J., Paek, K.Y., 2012. Sucrose regulated enhanced induction of anthraquinone, phenolics, flavonoids biosynthesis and activities of antioxidant enzymes in adventitious root suspension cultures of *Morinda citrifolia* (L.). Acta Physiol Plant 34(2), 405-415.

Beltrán, D., Selma, M.V., Marín, A., Gil, M.I., 2005. Ozonated water extends the shelf life of fresh-cut lettuce. J Agric Food Chem 53(14), 5654-5663.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': The FRAP assay. Anal Biochem 239(1), 70-76.

Borgognone, D., Marcucci, A., Colla, G., Renzaglia, M., Rea, E., Cardarelli, M., 2013. *In vitro* growth of *Aloe barbadensis* Mill.: the effect of activated charcoal on medium pH, nitrose uptake and elements content of shoots. Propagation Ornamental Plants 13(3), 138-145.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 86, 248-254.

Chakrabarty, D., Datta, S.K., 2008. Micropropagation of gerbera: lipid peroxidation and antioxidant enzyme activities during acclimatization process. Acta Physiol Plant 30(3), 325-331.

Chakrabarty, D., Dewir, Y.H., Hahn, E.J., Datta, S.K., Paek, K.J., 2007. The dynamics of nutrient utilization and growth of apple root stock 'M9 EMLA' in temporary versus continuous immersion bioreactors. Plant Growth Regul 51, 11-19.

Chakrabarty, D., Hahn, E.J., Yoon, Y.S., Paek, K.Y., 2003. Micropropagation of apple root stock 'M9 EMLA' using bioreactor. J Hortic Sci Biotechnol 78, 605-609.
Chakrabarty, D., Park, S.Y., Ali, M.B., Shin, K.S., Paek, K.Y., 2006. Hyperhydricity in apple: physiological and ultrastructural aspects. Tree Physiol 26, 377-388.

Chen, G.X., Asada, K ., 1989. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 30, 987-998.

Cui, X.H., Chakrabarty, D., Lee, E.J., Paek, K.Y., 2010. Production of adventitious roots and secondary metabolites by *Hypericum perforatum* L. in a bioreactor. Bioresource Techn 101, 4708-4716.

Debnath, S.C., 2008. Developing a scale-up system for the *in vitro* multiplication of thidiazuron-induced strawberry shoots using a bioreactor. Can J Plant Sci 88, 737-746.

De Oliveira, E.T., Crocomo, O.J., Farinha, T.B., Gallo, L.A., 2009. Large-scale micropropagation of *Aloe vera*. HortSci 44(6), 1675-1678.

Dewir, Y.H., Chakrabarty, D., Hahn, E.J., Paek, K.Y., 2005. Reversion of inflorescence in *Euphorbia milii* and its application to large scale micropopagation in an airlift bioreactor. J Hortic Sci Biotechnol 80, 581-587.

Dewir, Y.H., Chakrabarty, D., Hahn, E.J., Paek, K.Y., 2006. A simple method for mass propagation of *Spathiphyllum cannifolium* using an airlift bioreactor. In Vitro Cell Dev Biol - Plant 42, 291-297.

Escalona, M., Samson, G., Borroto, C., Desjardins, Y., 2003. Physiology of effects of temporary immersion bioreactors on micropropagated pineapple plantlets. In Vitro Cell Dev Biol - Plant 39, 651-656.

Fagnano, M., 2011. Effetti dell'inquinamento da ozono sulle colture ortofrutticole. Italus Hortus 18(3), 1-8.
Falkiner, F.R., 1998. The consequences of antibiotic use in horticulture. Journal of Antimicrobial Chemotherapy 41(4), 429-431.

Feng, Z., Niu, J., Zhang, W., Wang, X., Yao, F., Tian, Y., 2011. Effects of ozone exposure on sub-tropical evergreen *Cinnamomum camphora* seedlings grown in different nitrogen loads. Trees - Structure and Function 25(4), 617-625.

Folin, O., Ciocalteu, V., 1927. On tyrosine and tryptophan determination in proteins. J. Biol. Chem. 27, 627-650.

Gertlowski, C., Petersen, M., 1993. Influence of the carbon source on growth and rosmarinic acid production in suspension cultures of *Coleus blumei*. Plant Cell Tissue Org Cult 34(2), 183-190.

Gillespie, K.M., Rogers, A., Ainsworth, E.A., 2011. Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). J Exp Bot 62(8), 2667-2678.

Graham, T., Zhang, P., Woyzbun, E., Dixon, M., 2011. Response of hydroponic tomato to daily applications of aqueous ozone via drip irrigation. Sci Hortic 129, 464-471.

Gupta, S.D., Datta, S., 2004. Antioxidant enzyme activities during *in vitro* morphogenesis of gladiolus and the effect of application of antioxidants on plant regeneration. Biol Plant 47(2), 179-183.

Hahn, E.J., Paek, K.Y., 2005. Multiplication of *Chrysanthemum* shoots in bioreactors as affected by culture method and inoculation density of single node stems. Plant Cell Tissue Org Cult 81, 301-306.

Hashem Abadi, D., Kaviani, B., 2010. *In vitro* proliferation of an important medicinal plant *Aloe* - A method for rapid production. Australian J Crop Sci 4(4), 216-222.
Hassenberg, K., Idler, C., Molloy, E., Geyer, M., Plöchl, M., Barnes, J., 2007. Use of ozone in a lettuce-washing process: An industrial trial. J Sci Food Agric 87(5), 914-919.

Hazarika, B.N., 2006. Morpho-physiological disorders in *in vitro* culture of plants. Sci Hortic 108(2), 105-120.

Ho, C.W., Jian, W.T., Lai, H.C., 2006. Plant regeneration via somatic embryogenesis from suspension cell cultures of *Lilium X formolongi* Hort. using a bioreactor system. In Vitro Cell Dev Biol - Plant 42(3), 240-246.

Jo, E.A., Murthy, H.N., Hahn, E.J., Paek, K.Y., 2008. Micropropagation of *Alocasia amazonica* using semisolid and liquid cultures. In Vitro Cell Dev Biol - Plant 44(1), 26-32.

Jo, E.A., Tewari, R.K., Hahn, E.J., Paek, K.Y., 2009. *In vitro* sucrose concentration affects growth and acclimatization of *Alocasia amazonica* plantlets. Plant Cell Tissue Org Cult 96(3), 307-315.

Kämäräinen-Karppinen, T., Virtanen, E., Rokka, V.M., Pirttilä, A.M., 2010. Novel bioreactor technology for mass propagation of potato microtubers. Plant Cell Tissue Org Cult 101(2), 245-24.

Kikowska, M., Budzianowski, J., Krawczyk, A., Thiem, B., 2012. Accumulation of rosmarinic, chlorogenic and caffeic acids in *in vitro* cultures of *Eryngium planum* L. Acta Physiol Plant 34(6), 2425-2433.

Klapheck, S., Zimmer, I., Cosse, H., 1990. Scavenging of hydrogen peroxide in the endosperm of *Ricinus communis* by ascorbate peroxidase. Plant Cell Physiol 31(7), 1005-1013.

Leifert, C., Ritchie, J., Waites, W.M., 1991. Contaminants of plant tissue and cell cultures. World J Microbiol Biotechnol 7, 452-469.

Lian, M.L., Chakrabarty, D., Paek, K.Y., 2003. Bulblet formation from bulbscales segments of *Lilium* using bioreactor system. Biol Plant 46(2), 199-203.

López-Berenguer, C., Martínez-Ballesta, M.D.C., Moreno, D.A., Carvajal, M., García-Viguera, C., 2009. Growing hardier crops for better health: Salinity tolerance and the nutritional value of broccoli. J Agric Food Chem 57(2), 572-578.

Luna, C., Acevedo, R., Collavino, M., González, A., Mroginski, L., Sansberro, P., 2013. Endophytic bacteria from *Ilex paraguariensis* shoot cultures: localization, characterization, and response to isothiazolone biocides. In Vitro Cell Dev Biol - Plant: 49, 326-332.

Luna, C., Collavino, M., Mroginski, L., Sansberro, P., 2008. Identification and control of bacterial contaminants from *Ilex dumosa* nodal segments culture in a temporal immersion bioreactor system using 16S rDNA analysis. Plant Cell Tissue Org Cult 95, 13-19.

Luna, C., Collavino, M., Sansberro, P., Mroginski, L., 2009. Bacterial contamination in *Ilex dumosa* (Aquifoliaceae) cultures: antibiotics treatment. Acta Hortic 812, 97-102.

Mitrović, A., Janošević, D., Budimir, S., Bogdanović Pristov, J., 2012 Changes in antioxidative enzymes activities during *Tacitus bellus* direct shoot organogenesis. Biol Plant 56(2), 357-361.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends in Plant Sci 7(9), 405-410.

Mohamed, M.A.H., Ibrahim, T., 2011. *In vitro* mass production of *Ruta gravoelens* L. For secondary products production. Acta Physiol Plant 33, 1945-1951.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15, 473-497.

Natali, L., Sanchez, I.C., Cavallini, A., 1990. *In vitro* culture of *Aloe barbadensis* Mill.: Micropropagation from vegetative meristems. Plant Cell Tissue Org Cult 20(1), 71-74.

Niemenak, N., Noah, A.M., Omokolo, D.N., 2013. Micropropagation of cocoyam (*Xanthosoma sagittifolium* L. Schott) in temporary immersion bioreactor. Plant Biotech Rep 7, 383-390.

Oueslati, S., Karray-Bouraoui, N., Attia, H., Rabhi, M., Ksouri, R., Lachaal, M., 2010. Physiological and antioxidant responses of *Mentha pulegium* (Pennyroyal) to salt stress. Acta Physiol Plant 32(2), 289-296.

Paek, K.Y., Chakrabarty, D., Hahn, E.J., 2005. Application of bioreactor systems for large scale production of horticultural and medicinal plants. Plant Cell Tissue Org Cult 81, 287-300.

Paoletti, E., 2009. Ozone and mediterranean ecology: plants, people, problems. Environmental Pollution 157(5), 1397-1398.

Pati, P.K., Kaur, J., Singh, P., 2011. A liquid culture system for shoot proliferation and analysis of pharmaceutically active constituents of *Catharanthus roseus* (L.) G. Don. Plant Cell Tissue Org Cult 105, 299-307.

Piao, X.C., Charkrabarty, D., Hahn, E.J., Paek, K.Y., 2003. A simple method for mass production of potato microtubers using a bioreactor system. Curr Sci 8, 1129-1232.

Radhakrishnan, R., Lee, I.J., 2013. Ameliorative effects of spermine against osmotic stress through antioxidants and abscisic acid changes in soybean pods and seeds. Acta Physiol Plant 35(1), 263-269.

Rajeswari, V., Paliwal, K., 2008. Peroxidase and catalase changes during *in vitro* adventitious shoot organogenesis from hypocotyls of *Albizia odoratissima* L.f. (Benth). Acta Physiol Plant 30(6), 825-832.

Roels, S., Escalona, M., Cejas, I., Noceda, C., Rodriguez, R., Canal, M.J., Sandoval, J., Debergh, P., 2005. Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tissue Org Cult 82, 57-66.

Russowski, D., Maurmann, N., Rech, S.B., Fett-Neto, A.G., 2006. Role of light and medium composition on growth and valepotriate contents in *Valeriana glechomifolia* whole plant liquid cultures. Plant Cell Tissue Org Cult 86, 211-218.

Sáez, P.L., Bravo, L.A., Latsague, M.I., Sánchez, M.E., Ríos, D.G., 2012. Increased light intensity during *in vitro* culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Sci Hortic 138, 7-16.

Sajid, G.M., Pervaiz, S., 2008. Bioreactor mediated growth, culture ventilation, stationary and shake culture affects on *in vitro* growth of sugarcane. Pak J Bot 40(5), 1949-1956.

Scherer, R.F., Garcia, A.C., Fraga, H.P., Dal Vesco, L.L., Steinmacher, D.A., Guerra, M.P., 2013. Nodule cluster cultures and temporary immersion bioreactors as a high performance micropropagation strategy in pineapple (*Ananas comosus* var. *comosus*). Sci Hortic 151, 38-45.

Shaik, S., Dewir, Y.H., Singh, N., Nicholas, A., 2010. Micropropagation and bioreactor studies of the medicinally important plant *Lessertia* (Sutherlandia) *frutescens* L.. South African J Bot 76, 180-186.

Sharma, V., Goyal, S., Ramawat, K.G., 2011. Increased puerarin biosynthesis during *in vitro* shoot formation in *Pueraria tuberosa* grown in growtek bioreactor with aeration. Physiol Mol Biol Plants 17(1), 87-92.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K., 2002. Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot 53(372), 1305-1319.

Shohael, A.M., Chakrabarty, D., Ali, M.B., Yu, K.W., Hahn, E.J., Lee, H.L., Paek, K.Y., 2006. Enhancement of eleutherosides production in embryogenic cultures of *Eleutherococcus sessiliflorus* in response to sucrose-induced osmotic stress. Process Biochem 41, 512-518.

Sivakumar, G., Kim, S.J., Hahn, E.J., Paek, K.Y., 2005. Optimizing environmental factors for large-scale multiplication of chrysanthemum (*Chrysanthemum grandiflorum*) in balloon-type bioreactor culture. In Vitro Cell Dev Biol - Plant 41, 822-825.

Sreedhar, R.V., Venkatachalam, L., Thimmaraju, R., Bhagyalakshmi, N., Narayan, M.S., Ravishankar, G.A., 2008. Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. Biol Plant 52, 355-360.

Thanh, N.T., Murthy, H.N., Yu, K.W., Jeong, C.S., Hahn, E.J., Paek, K.Y., 2006. Effect of oxygen supply on cell growth and saponin production in bioreactor cultures of *Panax ginseng*. J Plant Physiol 163, 1337-1341.

Thind, S.K., Jain, N., Gosal, S.S., 2008. Micropropagation of *Aloe vera* L. and estimation of potentially active secondary constituents. Phytomorphology: An International J Plant Morphology 58(1-2), 65-71.

Vatankhah, E., Niknam, V., Ebrahimzadeh, H., 2010. Activity of antioxidant enzyme during *in vitro* organogenesis in *Crocus sativus*. Biol Plant 54(3), 509-514.

Wang, X., Zhang, Q., Zheng, F., Zheng, Q., Yao, F., Chen, Z., Zhang, W., Hou, P., Feng, Z., Song, W., Feng, Z., Lu, F., 2012. Effects of elevated O_3 concentration on winter wheat and rice yields in the Yangtze River Delta, China. Environmental Pollution 171, 118-125.

Wu, C.H., Dewir, Y.H., Hahn, E.J., Paek, K.Y., 2006. Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of *Echinacea angustifolia*. J. Plant Biol. 49, 193-199.

Wu, S.Q., Lian, M.L., Gao, R., Park, S.Y., Piao, X.C., 2011. Bioreactor application on adventitious root culture of *Astragalus membranaceus*. In Vitro Cell Dev Biol - Plant 47, 719-724.

Xu, H., Chen, S.B., Biswas, D.K., Li, Y.G., Jiang, G.M., 2009. Photosynthetic and yield responses of an old and a modern winter wheat cultivars to short-term ozone exposure. Photosynthetica 47(2), 247-254.

Yan, H., Lian, C., Li, Y., 2010. Improved growth and quality of *Siraitia grosvenorii* plantlets using a temporary immersion system. Plant Cell Tissue Org Cult 103, 131-135.

Yang, J.F., Piao, X.C., Sun, D., Lian, M.L., 2010. Production of protocorm-like bodies with bioreactor and regeneration *in vitro* of *Oncidium* 'Sugar Sweet'. Sci Hortic 125, 712-717.

Yonghua, Q., Shanglong, Z., Asghar, S., Lingxiao, Z., Qiaoping, Q., Kunsong, C., Changjie, X., 2005. Regeneration mechanism of Toyonoka strawberry under different color plastic films. Plant Sci 168, 1425-1431.

Zhang, W.W., Niu, J.F., Wang, X.K., Tian, Y., Yao, F.F., Feng, Z.Z., 2011. Effects of ozone exposure on growth and photosynthesis of the seedlings of *Liriodendron chinense* (Hemsl.) Sarg, a native tree species of subtropical China. Photosynthetica 49(1), 29-36.

Zhang, L., Su Su, B.Y., Li, Y.G., 2012. Growth and photosynthetic responses of four landscape shrub species to elevated ozone. Photosynthetica 50(1), 67-76.

Zhang, L., Xu, H., Yang, J.C., Li, W.D., Jiang, G.M., Li, Y.G., 2010. Photosynthetic characteristics of diploid honeysuckle (*Lonicera japonica* Thumb.) and its autotetraploid cultivar subjected to elevated ozone exposure. Photosynthetica 48, 87-95.

2.4. MICROPROPAGATION OF *LILIUM* (LA) HYBRID "BRINDISI" BULBLET IN CONTINUOUS IMMERSION BIOREACTOR

2.4.1 ABSTRACT

The present study investigated the growth and physiological parameters for micropropagation of *Lilium* bulblets in a continuous immersion bioreactor. Two experiments were performed. Exp. 1. In order to avoid the high risk of contamination in bioreactor culture, was evaluated the effect of ozone (O_3) treatments (5,10,15 min of O_3 exposure per day) in the system. Exp. 2. The dynamic of *Lilium* bulblet growth for the optimum culture period. The results showed that after 40 days of ozone bioreactor culture, biomass accumulation of *Lilium* increased markedly. Moreover the contamination was zero during the whole culture period in bioreactors with O_3 exposure. Moreover the contamination was not detectable during the whole culture period in bioreactors with O_3 exposure. However between O_3 treatments fresh weight (total and per explant), growth index, number of shoots per explant, shoot diameter and number of roots per explant were significantly higher in bioreactors with 5 and 10 min of ozone. The activity of Catalase, Guaiacol peroxidase, lipid peroxidation and Chlorophyll pigments in bulblets no had significant difference between O_3 treatments. However, the highest values of ascorbate peroxidase, H_2O_2 and levels of FRAP in bulblets from bioreactor culture under high O_3 exposure per day, indicated low signs of oxidative stress in these bulblets. In the second experiment the dynamic of bulblet growth revealed that biomass accumulation increased exponentially from initial culture to 30 days, and then a low increase in biomass was observed from 30 to 40 days. Electrical conductivity of the residual medium showed a low increased of 5.7 to 5.9 mS cm^{-1} until the 20th day, and then EC gently decreased in the following days. The total

soluble solids was gradually increased from 6.8 to 10.3 during bioreactor culture. Changes of pH in culture medium exhibit a dramatically decreased from 5.8 to 3.9 on day 5 and then remained more or less constant during the whole period of growth. High decrease in pH at the beginning of culture may be explained by the ammonium uptake. NH_4^+ was rapidly depleted leading to higher acidification of the medium. The time course of the others macro- and micro-nutrients concentrations in the media showed also a rapid depletion of P as NH_4^+ and NO_3^-. The concentrations of NH_4^+, NO_3^-, Ca, Al, Cu and Mn in bublets are inversely correlated with concentrations in nutrient medium. The activity of CAT, G-POD and the lipid peroxidation in bulblets did not shown a significant differences during the whole culture period. However the activity of APX and FRAP, were significantly higher in bulblets from 20 to 40 days of culture and the production of H_2O_2 increased from initial culture until 40 days.

Keywords BTBB type - liquid medium - ozone sterilization - antioxidant enzymes - growth dynamic - nutrient utilization

2.4.2 INTRODUCTION

During the last 50 years lily has become one of the most important flowers bulbls and cut flowers in the world (Van Tuyl and Arens, 2011). The LA hybrid group was developed by interspecific hybridization between hybrids of different sections, the *Longiflorum* and the Asiatic group, and recently this group has became important in the industry of International Market bulbs (Van Tuyl and Arens, 2011). Lily has been successfully tissue cultured from bulb scale sections (Gupta et al., 1979; Nightingale, 1979; Stimart and Ascher, 1979, 1981; Novak and Petru, 1981;Takayama and Misawa, 1980, 1983, Han et al., 2004; Cardona Suarez 2011), and it is now the current commercial method for the production and formation of bulblets in the micropropagation of *Lilium* species, (Gerrits and De Klerk,1992; Priyadarshi and Sen, 1992; Tanimoto and Matsubara, Lian et al, 2003). However plant micropropagation required intensive tissue handling, periodic transfers of plant material to fresh media, due to exhaustion of the nutrients in the medium and also because of continuous tissue growth and proliferation, which is rapidly limited by the size of the culture container (Maene and Debergh, 1985). High tissue handling cost resulting in high production costs and this limit the commercial use of micropropagation to products with a very high unit value (Sluis and Walker, 1985; Simonton et al., 1991). Bioreactors have been

used for large scale micropropagation due to the possibilities of automation; scaling up of mass propagation, high control of culture factors such as physical and chemical environments, scheduled shipping and reduction of the production cost (peak et al., 2001). Accordingly mass propagation of plant tissue organs in *Lilium* by bioreactors has been examined by some authors (Takayama and Misawa,1983; Takahaski et al.; 1992; Tsumaki and Takayama, 1992; Lim et al., 1998; Seon et al., 2000, Kim et al., 2001, Lian et al., 2001, Lian et al., 2003a, Lian et al., 2003b, Ho et al., 2006, Barberine et al., 2011. Cardona Suarez et al., 2012). However a serious limited to bioreactor culture is contamination, the bacterial contamination by the explants may appear in just a few days, as well the risk of exogenous contamination is high and increases with the manual handling of sampling or adding medium. The conventional disinfection methods with sodium hypochlorite or antibiotics commonly used in micropropagation are not efficient to ensure a complete sterilization in the bioreactor, due to both the explant as exogenous contamination (Luna et al. 2009.; Cardona Suárez et al. 2012). In order to develop a feasible methodology to eliminate the high risk of contamination for mass commercial production of lily bulblets in bioreactor system, was investigated the effect of ozone (O_3) treatments in bioreactor culture of Lilium, due to Ozone, is a powerful oxidant principally applied as disinfectant of drinking and waste water (Sechi et al. 2001; Hassenberg et al., 2007). In addition was examined the dynamic of *Lilium* bulblet growth for the optimum culture period.

2.4.3 MATERIALS AND METHODS

2.4.3.1 Plant material

Scales from Bulbs of LA-Hybrid lilies (Brindisi ®) were aseptically excised and cut into 0.6 cm sections (Cardona Suárez et al., 2011). Each section of the bulb scale was cultured onto MS medium (0.7% agar) (pH 5.8), supplemented with 6% (w/v) sucrose, 4.4 µM (BA) and 1.1 µM (NAA). After five weeks bulblets obtained from the basal parts of bulb scale segments were randomly cut into sections of about 5–7 mm in size and used as a source of explants for this experiment (Fig.1).

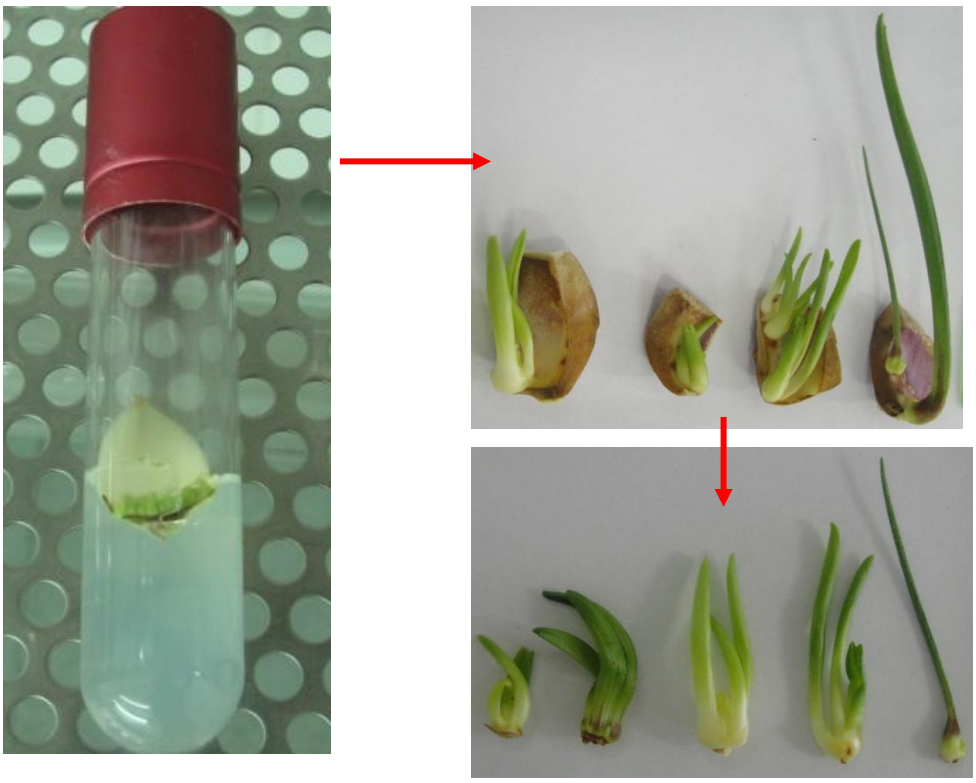


Fig. 1 Bulblets from bulb scale segments of LA-Hybrid lilies (Brindisi ®).

2.4.3.2 Bulblet culture in bioreactor

Bulblets regenerated in vitro (approx 25 bulblets per bioreactor) (10 g) were transferred to 4 L Balloon type bubble bioreactor (BTBB) (Fig. 2) containing 1.5 liters of MS liquid medium supplemented with 6% (w/v) sucrose, 4.4 µM (BA) and 1.1 µM (NAA). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C/103 kPa for 25 min. A supporting net was used to hold the plant material in order to avoid complete submersion of explants in the liquid medium. The volume of air input was adjusted to 0.1 vvm (air volume/culture volume min). Bioreactors were maintained at 25^{o}C±1°C in dark. Two different experiments were performed using bioreactor cultures.

2.4.3.3 Experiment 1. Effect of ozone treatments in bioreactor culture of *Lilium*

Three different treatments using ozone were investigated in bioreactor culture of lily bulblets; 5 min of O_3 per day, 10 min of O_3 per day and 15 min of O_3 per day were applied in the bioreactors for the entire period of the culture (40 days). Ozone was generated by corona discharge (ozone tube) using ambient air with an O_3 generator (Model OZX-300AT ENALY M&E, Shanghai, China) having a maximum ozone output of 200 m h^{-1} at a flow rate of 1-2 l min^{-1}. Ozone has been made to flow inside the continuous immersion bioreactors together with the air inlet.

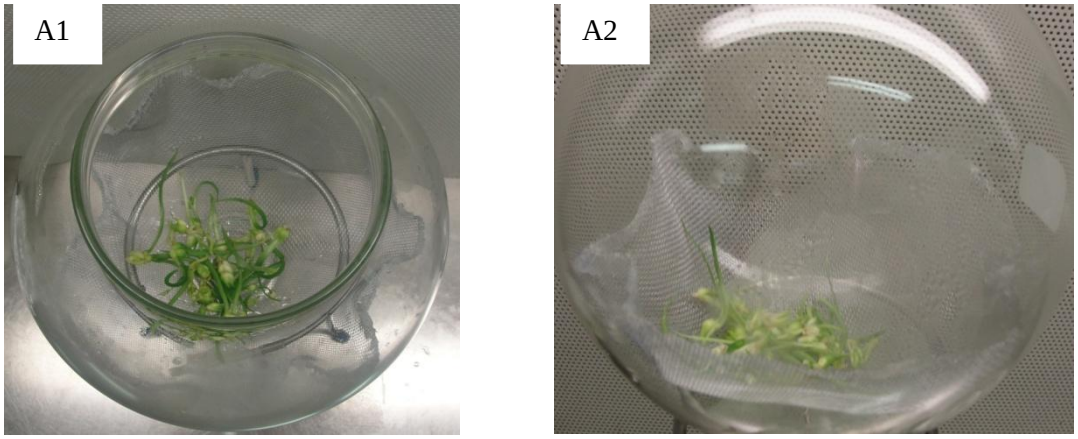


Fig. 2 A1-A2 Inoculum of Bulblet explants of LA-Hybrid lilies (Brindisi ®) in bioreactor system.

2.4.3.4 Experiment 2. The optimum culture period

To determine the dynamic of *Lilium* bulblet growth, the bulblets were harvested from the bioreactor at 10 days intervals for 40 days, and changes in pH, EC total soluble solids (TSS), Macro- and micro-nutrients in medium were determined during bioreactor culture. In this experiment 5 minutes of O_3 per day was applied during the whole culture period.

2.4.3.5 Determination of pH, EC, total soluble solids (TSS), in spent media

In the second experiment, samples (20 ml) were collected at an interval of 5 days up to 40 days of bioreactor culture through the sampling port. pH and EC of the medium were measured using a pH meter (GLP21, Crison Instruments, Barcelona, Spain) and EC meters (HI 86304, Hanna Instruments, Padova, Italy), respectively. TSS content was determined by a digital refractometer (PDR 30, XS Instruments, Poncarale, BS, Italy) and expressed as Brix at 20ºC. Media were then stored at –80ºC until analyzed.

2.4.3.6 Determination of Macro- and micro-nutrients in shoots tissues and spent media

In the second experiment, dried shoots taken at the end of culture were ground separately in a Wiley mill to pass through a 20-mesh screen, then 0.5 g of the dried shoots were analyzed for the following macro- and micro-nutrients: N, P, K, Ca, Mg, Na, B, Al, Cu, Fe and Mn. Nitrogen as Nitrate (NO_3^-) and Ammonium (NH_4^+) concentrations were determinated in shoots using the salicylic sulfuric acid method as described by Cataldo *et al*. (1975), and the colorimetric method as described by

Anderson and Ingram (1989) respectively. P, K, Ca, Mg, Na, B, Al, Cu, Fe and Mn concentrations were determined by dry ashing at 400^{o}C for 24 h, dissolving the ash in 1:25 HCl, and assaying the solution obtained using an inductively coupled plasma emission spectrophotometer (ICP Iris; Thermo Optek, Milano, Italy) (Karla, 1998). The concentrations of various elements in shoots were expressed in mg kg^{-1} DW. For estimation of Macro-and micro-nutrients in spent media , the medium were thawed and passed sequentially through 0.2 µm membrane filter for particulate removal, and 10 ml of solution obtained was used for mineral analysis using the same methods as described above. The concentrations of various elements in media were expressed in mg l^{-1}.

2.4.3.7 Measures

Several growing parameters were recorded at the end of each experiment. Fresh and dry weight of bulblets, number of shoots, shoot length, shoot diameter, and number of roots per explant, were measured after rinsing once with sterile water and blotting away the surface water. Dry weight was determined after drying for 48 h at 70°C. Growth ratio was calculated as described by Ziv (2005) [Growth value = (Final fresh weight (g)-Initial fresh weight)/ initial fresh weight]. A part of the plant material was immediately frozen in liquid nitrogen and maintained at -80 °C until analyzed.

2.4.3.8 Antioxidant enzyme assay

Enzyme extractions were performed using a pre-chilled mortar and pestle with two volumes of an ice-cold extraction buffer (0.05 M potassium phosphate buffer, pH 7.0) containing 0.1% (w/v) ascorbic acid, 1% (w/v) polyvinilpolypirrolidone (PVPP), 1 mMNa_2-EDTA and 0.1% (v/v) Triton X-100. Extracts were centrifuged at 15000 *g* for 10 min at 4 ºC and the supernatants were used for the determination of the enzymes activity and protein content by a spectrophotometer (Helios Beta, Spectrophotometer, Thermo Electron Corporation, England). The protein concentration of the enzyme extract was determined according Bradford method (1976) using bovine serum albumin as standard.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined in 1 ml reaction mixture containing 50 mM K-phosphate (pH 7.0), 0.1 mM ascorbate (extinction coefficient, 2.8 mM^{-1} cm^{-1}), 0.3 mM H_2O_2. The decrease in absorbance was recorded at 290 nm for 1 min (Chen and Asada 1989) corresponding to the oxidation of ascorbic acid.

Catalase (CAT, EC 1.11.1.6) activity was directly measured by the decomposition of H_2O_2 (extinction coefficient, 0.036 mM^{-1} cm^{-1}) at 240 nm for 1 min in 1 ml assay mixture containing 50 mM K-phosphate buffer (pH 7.0), 125 mM H_2O_2 and 20 µl of the crude extract (Havir and McHale 1987).

2.4.3.9 Determination of Lipid peroxidation (MDA) content

Lipid peroxidation was estimated as the amount of the thiobarbituric acid-reactive substances (TBARS; mainly MDA from lipid peroxidation) determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). Fresh frozen sample was ground in a pre-chilled mortar, and homogenized in three volumes of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12 000 × *g* for 15 min at 4°C and 0.4 ml of supernatant was added to 0.8 ml 0.5% (w/v) thiobarbituric acid (TBA) in 20 % (w/v) TCA. The mixture was incubated at 95 °C for 30 min and then quickly cooled on ice. Samples were centrifuged at 10000 × *g* for 10 min at 4°C and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.4.3.10 Determination of hydrogen peroxide H_2O_2

Hydrogen peroxide levels were determined according to Sergiev et al. (1997). One gram of bulblets was homogenized in 3 ml of 0.1% (w/v) trichloroacetic acid in a pre-chilled mortar and pestle by liquid nitrogen. The homogenate was centrifuged at 12.000 g for 30 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM K-phosphate buffer (pH 7.0), and 1 ml reagent (1 M KI, w/v in fresh double-distilled water). The blank probe consisted of 10 mM K-phosphate buffer in the absence of extract. After 1 h of reaction in darkness, the absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H2O2.

2.4.3.11 FRAP (ferric reducing-antioxidant power) assay

The FRAP assay was carried out according to Benzie and Strain (1999) using a spectrophotometer (Helios Beta, Thermo Electron Corporation, England), the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol iron(III) chloride solution in the ratio 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily. Thirty microliters of sample were added to 1.0

mL of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 30-min incubation at 37°C. The standard curve was constructed using iron(II) sulfate solution, and the results were expressed as mmol Fe(II)/g fresh weight of plant material.

2.4.3.12 Measurements of chlorophyll and carotenoid contents

Samples (1 g) were prepared powdering shoots in a mortar and extracted with 10 ml of 80% (v/v) cold acetone. The resulting extract was centrifuged for 20 min at 4800×*g*. The pigment determination was spectophotometrically performed, measuring light absorbance by acetone extracts of chlorophylls a and b (663nm and 646nm) respectively, and carotenoids (470 nm). All amounts were expressed as µg per gram of fresh weight.

2.4.3.13 Experimental design and data analysis

The results shown are the mean values of two independent experiments. Experiments were set up in a completely randomized design and four replications were used in each treatment. Data were subjected to ANOVA and Duncan's multiple range test using SPSS program (Version 16, SAS Institute Inc., Cary, NC, USA).

2.4.4 RESULTS AND DISCUSSION

2.4.4.1 Experiment 1. Effect of ozone treatments in bioreactor culture of *Lilium*

Effect of ozone treatments on biomass accumulation.

After 40 days of bioreactor culture, biomass accumulation of *Lilium* (LA) hybrid "Brindisi" increased markedly in all ozone treatments (Table 1) 5, 10 and 15 min of O_3 per day resulting in optimum values of total fresh weight (190.6, 177.2 and 140.3) respectively. Similar responses of biomass accumulation was reported by Lian et al. 2003 in bioreactor culture of *Lilium* without O_3 exposure. Cardona et al. (2012*)* found lower values on growth of *lilium* (LA) hybrid "Brindisi" in bioreactor cultures with sterilization by hypochlorite and antibiotics. Moreover the contamination was not detectable during the whole culture period in bioreactors with O_3 exposure. Analogous results were reported in ozone bioreactor culture of *Aloe barbadensis*. These results indicate that ozone sterilization in bioreactor culture is effective to reduce and eliminate

the high risk of contamination, and assayed O_3 treatments no had elevated impacts on biomass accumulation. However between O_3 treatments fresh weight (total and per explant), growth index, number of shoots per explant, shoot diameter and number of roots per explant were significantly higher in bioreactors with 5 and 10 min of ozone. Nevertheless shoot length and dry matter no had significant differences between treatments (Table 1). Additionally no visible O_3 damage symptoms were observed in bulblets from bioreactors culture with 5 and 10 min of ozone sterilization, although a limited O_3 injury symptoms as spots in the tissue were observed in bulblets from bioreactor with 15 min of O_3 exposure per day (Fig. 3).

Effect of ozone treatments on stress levels, antioxidant properties and chlorophyll pigments in bioreactor culture of Lilium

The activity of Catalase (CAT), Guaiacol peroxidase (G-POD) and lipid peroxidation malonyldialdehyde - MDA content in bulblets did not shown a significant difference between O_3 treatments. However, activity of ascorbate peroxidase (APX), production of H_2O_2 and levels of FRAP (ferric reducing-antioxidant power) were significantly higher in bulblets from bioreactor culture with high O_3 exposure (15 min per day) and lower in bulblets grown in bioreactor with 5 and 10 min of O_3 exposure per day (Table 2). The formation of H_2O_2 is an important phenomenon of signal transduction induced by stress conditions, which changes the content redox agents and the redox state of cells (Jabs et al. 1997). H_2O_2 may function as a signal for induction of defense systems and could enhance secondary metabolite production (Berglund and Ohlsson 1995; Cui et al. 2010)). These results indicate that high O_3 exposure 15 min per day induced low oxidative stress in bulblets as indicated by an enhanced of levels of H_2O_2, FRAP and the activity of APX. In fact, the ozone penetrates inside of foliar tissues through the stomatal apertures. Once penetrated within the plants, due of its high oxidizing power (of secondary products derived by ozone, such as ROS) activates the synthesis of antioxidant compounds to detoxify the ROS (Sunkar et al., 2003; D'Hasse et al. 2005).

Chlorophyll, carotenoid contents, and Chl a b^{-1} ratio did not shown significant differences between ozone treatments (Table 2). These results indicate that ozone sterilization in bioreactor culture no had significant effects in pigment contents. However Xu et al. (2009); Feng et al. (2011); Zhang et al. b (2012) reported that O_3 exposure in plants reduced Chlorophyll and carotenoid contents

Table. 1. Effect of ozone treatments on shoot growth in bulblet culture of *Lilium* (LA) hybrid "Brindisi" after 40 days in a bioreactor.

Parameters	O5	O10	015	
Total fresh weight (g)	190.6 ± 5.44a	177.2 ± 3.53a	140.3 ± 4.92b	**
Growth value	18.06 ± 0.54a	16.72 ± 0.35a	13.03 ± 0.49b	**
Shoots explants^{-1} (n)	2.10 ± 0.29a	1.99 ± 0.16a	1.60 ± 0.30b	*
Fresh weight explant^{-1} (g)	8.53 ± 1.17a	7.49 ± 0.15ab	6.49 ± 0.36b	*
Shoot length (cm)	16.77 ± 1.65	16.21 ± 1.58	15.16 ± 0.31	NS
Shoot diameter (cm)	1.26 ± 0.06a	1.11 ± 0.05b	1.07 ± 0.06b	*
Dry matter (%)	13.73 ± 0.85	12.07 ± 1.53	13.29 ± 0.48	NS
Roots explant^{-1} (n)	5.01 ± 0.26a	5.46 ± 0.25a	2.49 ± 0.97b	***

O5: 5 min ozone per day; O10: 10 min ozone per day; O15: 15 min ozone per day. Supplying ozone: 200 mg h^{-1} at a flow rate of: 1-2 L min^{-1}

Means separation by Duncan's multiple range test, $P \leq 0.05$. NS, *, **, *** Non Significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively

Table. 2. Effect of ozone treatments on antioxidant enzyme activities and chlorophyll pigments in bulblet culture of *Lilium* (LA) hybrid "Brindisi" after 40 days in a bioreactor.

Parameters	O5	O10	015	
Ascorbate peroxidase (mmol min^{-1} mg^{-1} protein)	1.29 ± 0.15b	1.42 ± 0.07b	1.93 ± 0,39a	*
Catalase (mmol min^{-1} mg^{-1} protein)	69.8 ±2.3	63.1 ± 9.5	74.5 ± 14.2a	NS
Guaiacol peroxidase (mmol min^{-1} mg^{-1} protein)	1.59 ± 0.33	1.48 ± 0.09	1.53 ± 0.14	NS
Malonyldialdehyde (nmol g^{-1} FW)	2.92 ± 0.25	3.37 ± 0.26	3.65 ± 0.36	NS
FRAP assay (mmol Fe^{2+} g^{-1} FW)	7.77 ± 0.50b	7.60 ± 0.40b	9.54 ± 0.79a	*
Hydrogen peroxide (µmol g^{-1} FW)	469.1 ± 61.4b	549.2 ± 18.9b	734.4 ± 66.5a	*
Chlorophyll a (µg g^{-1} FW)	59.1 ± 4.92	63.0 ± 2.12	66.0 ± 2.59	NS
Chlorophyll b (µg g^{-1} FW)	29.7 ± 1.82	31.5 ± 0.78	31.9 ± 1.40	NS
Chlorophyll tot (µg g^{-1} FW)	88.8 ± 6.69	94.5 ± 2.52	98.0 ± 3.98	NS
Chlorophyll a/b	1.99 ± 0.06	2.00 ± 0.07	2.07 ± 0.02	NS
Carotenoids (µg g^{-1} FW)	15.9 ± 1.48	15.5 ± 0.59	17.7 ± 0.57	NS

O5: 5 min ozone per day; O10: 10 min ozone per day; O15: 15 min ozone per day. Supplying ozone: 200 mg h^{-1} at a flow rate of: 1-2 L min^{-1}

Means separation by Duncan's multiple range test, $P \leq 0.05$. NS, *, **, *** Non Significant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively

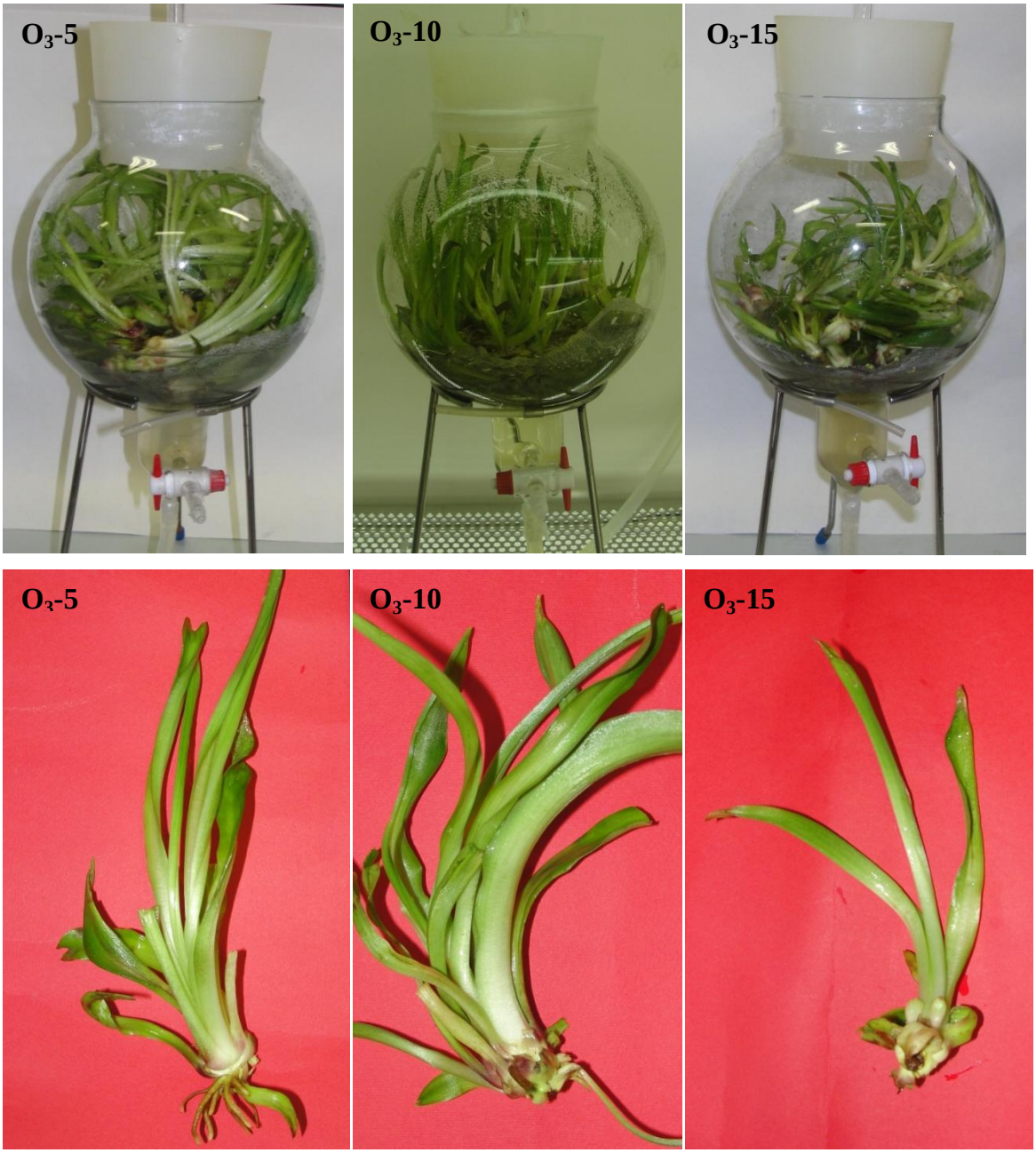


Fig.3 Bulblet growth of *Lilium* (LA) hybrid "Brindisi" by different ozone treatments after 40 days of bioreactor culture.

O_3-5: 5 min ozone per day; O_3-10: 10 min ozone per day; O_3-15: 15 min ozone per day

2.4.4.2 Experiment 2. The optimum culture period

Dynamic of Bulblet growth, EC, TSS, Ph and macro- and micro-nutrients in the medium during bioreactor culture

The dynamic of bulblet growth during bioreactor culture of *Lilium* (LA) hybrid "Brindisi" revealed that biomass accumulation increased exponentially from initial culture to 30 days, reached a values of 208.5 g of total FW and 8.32 g of FW per explant at the 30th day, and then a low increase in biomass was observed from 30 to 40 days, reached a values of 220 g and 8.32 g of total FW and FW per explant respectively. The others growth parameters as shoot length, shoot diameter and number of roots per explants increased proportionally from to 0 to 40 days without reaching peaks in the growth curve, however; the percentage of dry weight showing a lag phase from initial culture to 30 days, followed by an increase of 28% until 40 days, compared with the response at the 30th day (Fig 4 and 10). The lower dry mass of the bulblets obtained from 0 to 30 days, indicated that the bulblets contained more water as compared to those from 40 days. Earlier studies of bulblet growth of *Lilum* in bioreactor culture (Lian et al. 2002; Lian et al. 2003) indicate that bulblets grew faster when the medium was exchanged frequently with the new medium after 2 or 4 weeks of bioreactor culture in order to maintain high levels of sucrose in the medium, that fact may explain the lag phase of growth from 30 to 40 days, due to the initial medium was not renewed.

Electrical conductivity (EC) of the residual medium showed a low increased of 5.7 to 5.9 mS cm^{-1} until the 20th day, and then EC gently decreased in the following days (Fig 6). The total soluble solids (TSS) was gradually increased from 6.8 to 10.3 (^{0}Brix) during bioreactor culture. Changes of pH in culture medium exhibit a dramatically decreased from 5.8 to 3.9 on day 5 and then remained more or less constant during the whole period of growth (Fig 5). Similar responses of acidification of the medium in the first days of culture were reported for some authors (Lian et al. 2002; Shin et al. 2003; Yan et al. 2010).

In our study, a high decrease in pH at the beginning of culture may be explained by the ammonium uptake, NH_4^+ was rapidly depleted leading to higher acidification of the medium as shown in Fig. 6 (Chakrabarty et al., 2007).

This preferential uptake of NH_4^+ at the beginning of culture has been observed for many species (Shin et al., 2002; Jeong et al., 2006, 2009; Cui et al. 2010b) which is also associated with fall in pH of the culture media. This rapid decrease in pH of the culture media may be due to the efflux of protons during the absorption of NH_4^+ (Dantas et al., 2001; Lian et al., 2002; Chakrabarty et al., 2007). In addition, there was no significant uptake of NO_3^- before day 20 considerable uptake of NO_3^- started from day 20 of culture (Fig. 7) These results indicated that NH_4^+ uptake acidifies the medium and thus indirectly promotes NO_3^-uptake (Lian et al., 2002; Shin et al., 2003; Chakrabarty et al., 2007; Cui et al., 2010b).

Moreover, the time course of the others macro- and micro-nutrients concentrations (mg l^{-1}) in the media showed also a rapid depletion of P as NH_4^+ and NO_3^- (Fig. 6), however others elements such as K, Ca, Mg, Na, B, Fe, Cu, and Mn revealed a slowly increase during the initial period of growth and then remained more or less constant during the complete period of culture (Shin et al., 2003). These results indicate that increase of macro and micro-nutrients in the medium at the end of the culture was probably due to the interaction with plant tissue in bioreactor culture. Na uptake started from inoculation but after 15 days of culture also showed a slowly increase (Fig. 7). At the beginning of culture Aluminium concentration was zero, but the element increased with depletion of NH_4^+, NO_3^- and rapid fall in pH (5.8 to 3.9 after 5 days of culture) indicating exchangeable aluminium due to acidification of the medium.

There is a good correlation between availability of P and NH_4^+ in growth of bulblets in bioreactor culture of *Lilium* (Lian et al., 2002), Phosphorus and ammonium deficiency in the medium resulted in poor shoot growth and proliferation in continuous immersion culture of apple (Chakrabarty et al., 2007) and during hairy root growth of *Beta vulgaris* L. in liquid culture greater absorption of phosphate could be due to initiation of cell division of the explants (Shin et al., 2003). The fast depletion of NH_4^+, NO_3^- and P in our experiments may explain the slow phase of growth from 30 to 40 days of culture.

Thus it is a hypothesized that a cascade of events, including rapid depletion of P, NH_4^+ and NO_3^-, high decrease in pH, and exchangeable aluminium due to acidification of the medium, may be involved in the low increase in biomass accumulation of *Lilium* from 30 to 40 days of bioreactor.

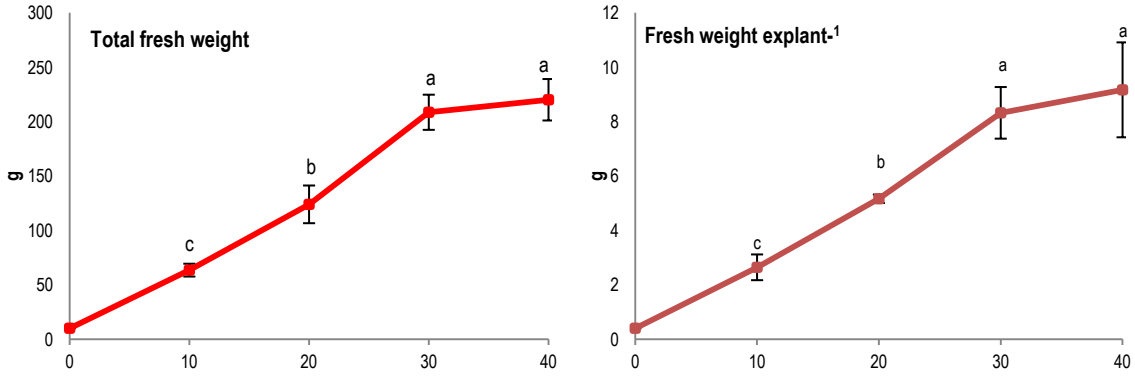
***Dynamic of* Macro- and micro-nutrients in shoots tissues and spent media**

The differential uptake of macro- and micro-elements in nutrient medium and bulblets were monitored continuously during the culture cycle (Fig. 7). The concentrations of NH_4^+, NO_3^-, Ca, Al, Cu and Mn in bublets are inversely correlated with concentrations in nutrient medium, initial uptake of these elements by bulblets increased with decreasing the mineral concentrations in the culture mediun. While the uptake of P, K and B between bulblets and nutrient medium showed a direct correlation, consumption of nutrients by bulblets increased with increasing the elements in culture medium. However, Mg, Na and Fe uptake between bulblets and nutrient medium did not show a significant correlation.

2.4.5 CONCLUSIONS

This study indicated that the use of ozone in bioreactor culture of Lilium by O_3 regenerator was an efficient instrument to reduce and eliminated the high risk of contamination of production in the liquid medium. and is a viable and efficient model for automation and large-scale production of quality shoots. In adittion results clearly suggest that a 4 l balloon type bubble bioreactor system cultured for 30 days was optimum culture period.

Changes on Bulblet growth during bioreactor culture of Lilium (LA) hybrid "Brindisi"



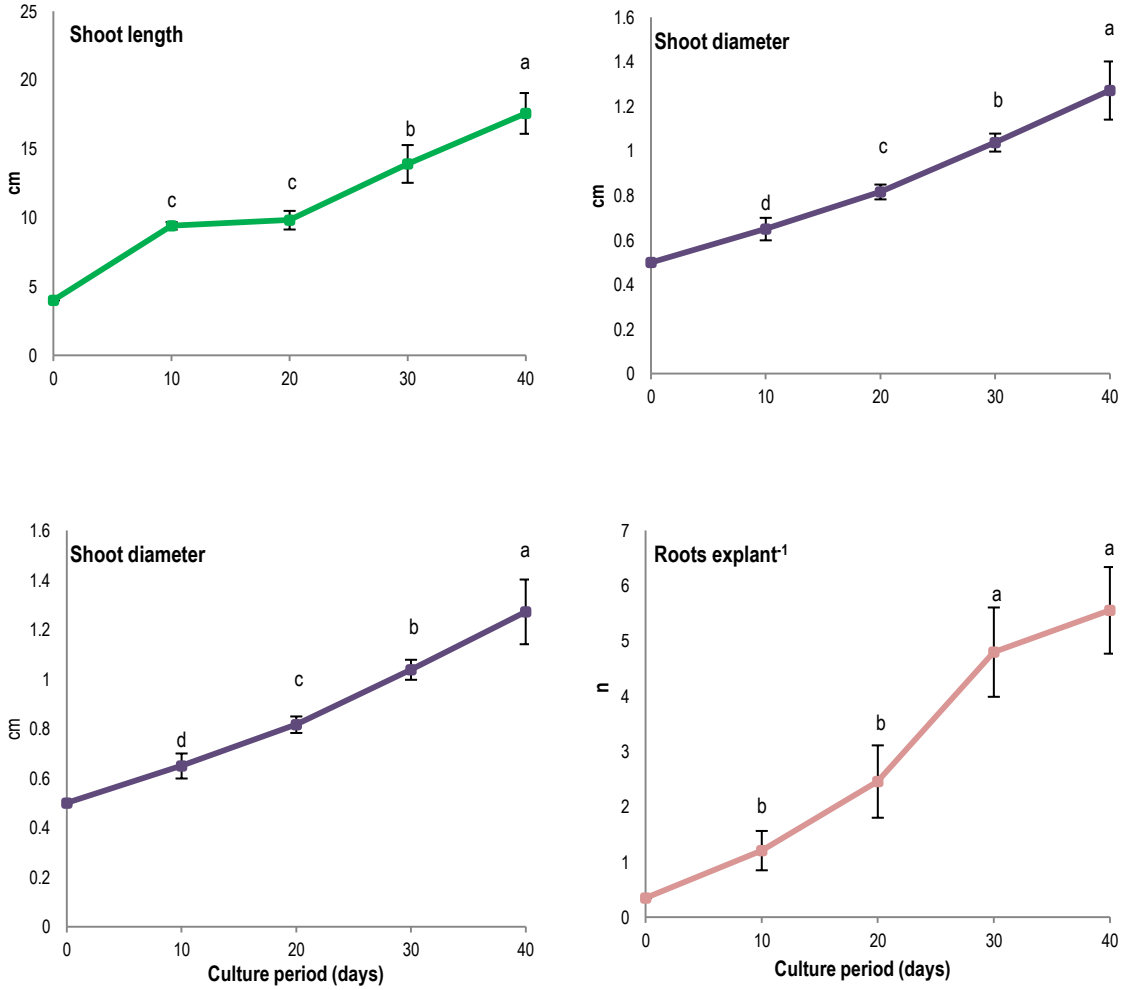


Fig 4. Time-course changes on bulblet growth during bioreactor culture of *Lilium* (LA) hybrid "Brindisi"

Changes in the pH, NH_4^+ uptake, EC and TSS in the nutrient medium, during bioreactor culture of Lilium (LA) hybrid "Brindisi"

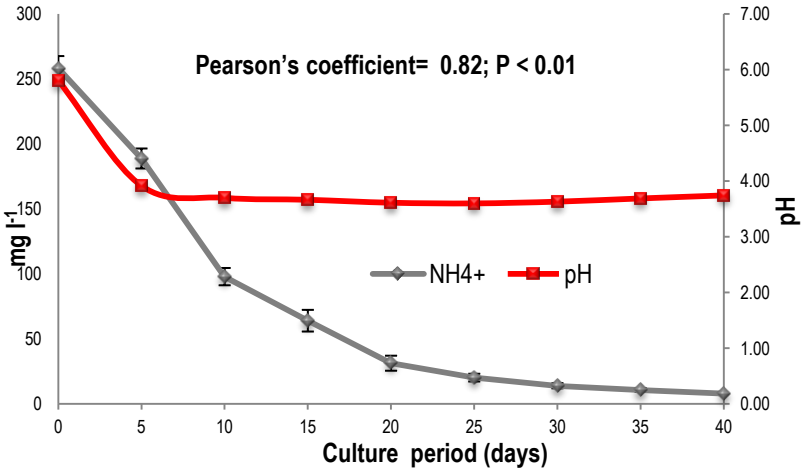


Fig. 5. Time course of changes and Pearson's correlation test between pH and NH^{4+} uptake in the nutrient medium during bioreactor culture of *Lilium* (LA) hybrid "Brindisi"

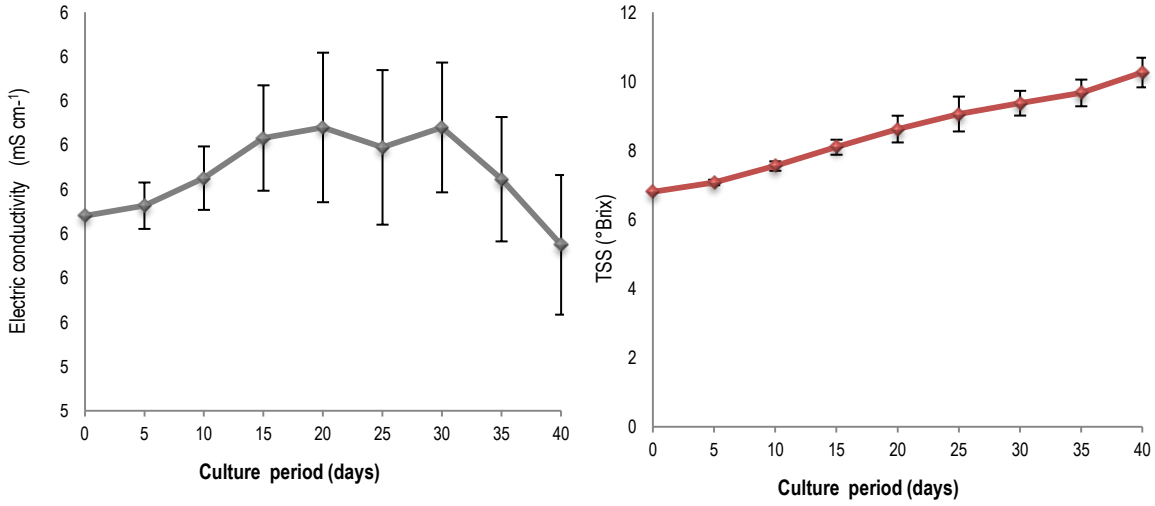
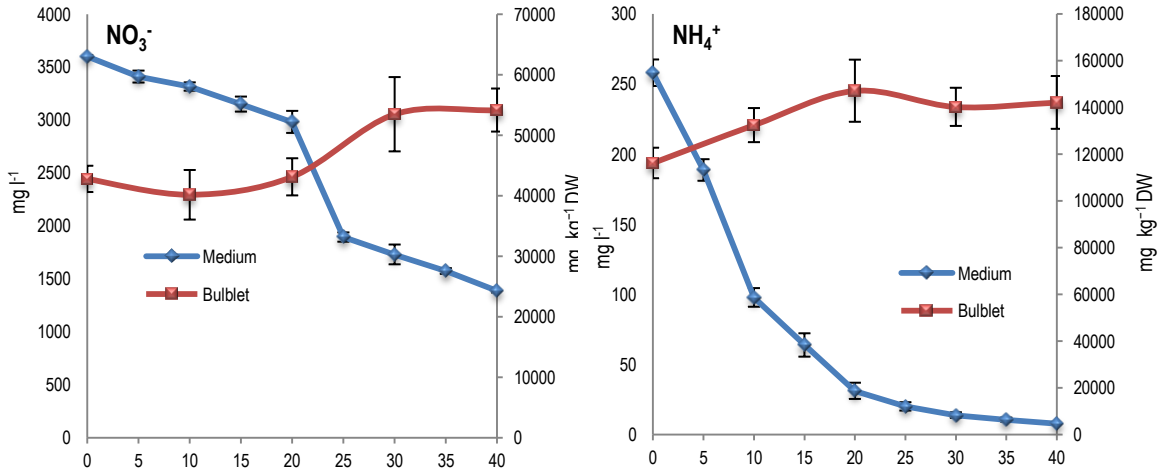
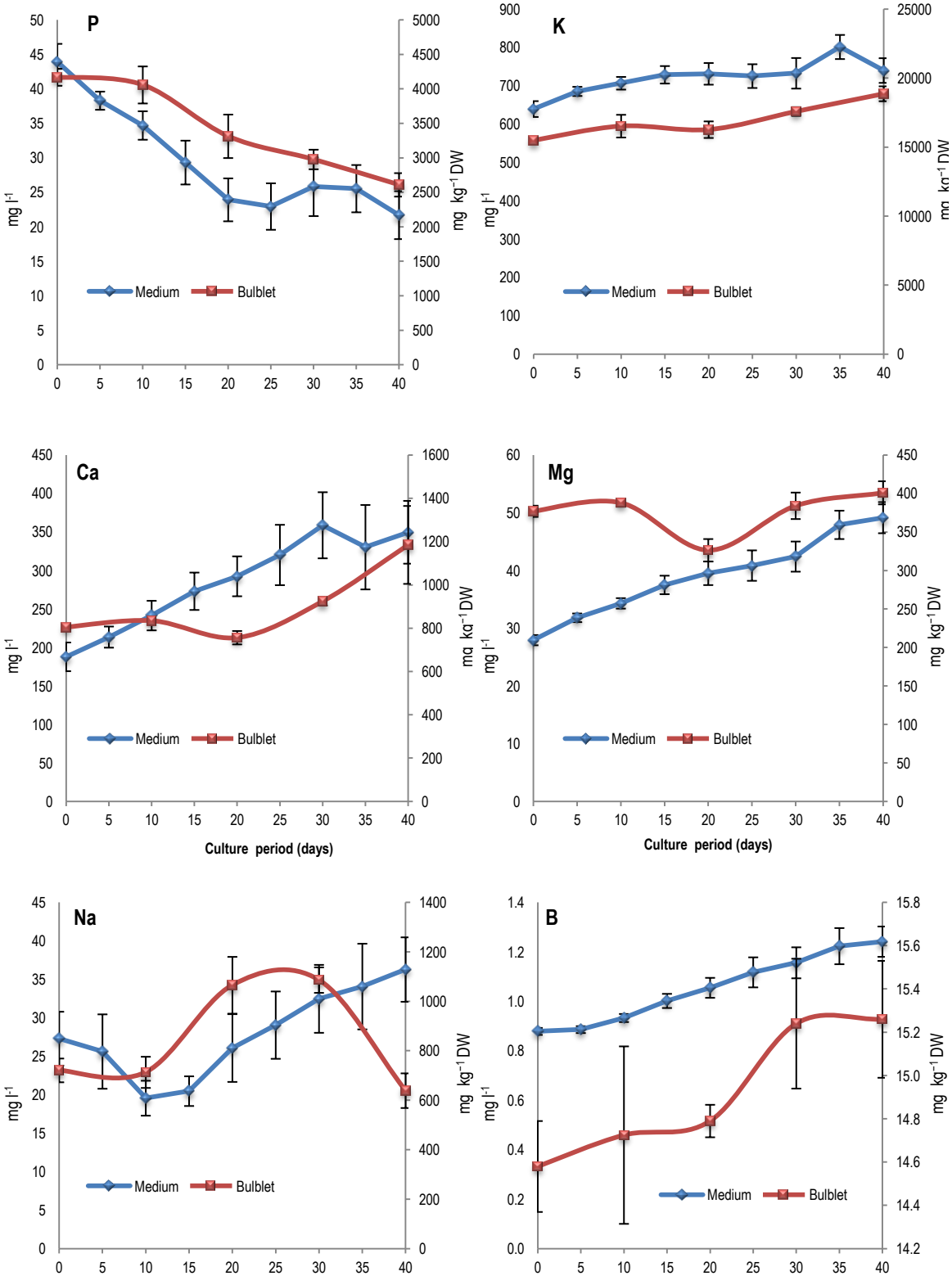


Fig 6. Time-course changes in EC and TSS of the nutrient medium, during bioreactor culture of *Lilium* (LA) hybrid "Brindisi"

Time-course changes on Macro-and micro-nutrients composition in bulblets and nutrient medium during bioreactor culture of Lilium (LA) hybrid "Brindisi"




P
mg l-1
mg kg-1 DW
Medium
Bulblet
K
mg l-1
mg kg-1 DW
Medium
Bulblet
Ca
mg l-1
mg kg-1 DW
Medium
Bulblet
Culture period (days)
Mg
mg l-1
mg kg-1 DW
Medium
Bulblet
Culture period (days)
Na
mg l-1
mg kg-1 DW
Medium
Bulblet
B
mg l-1
mg kg-1 DW
Medium
Bulblet

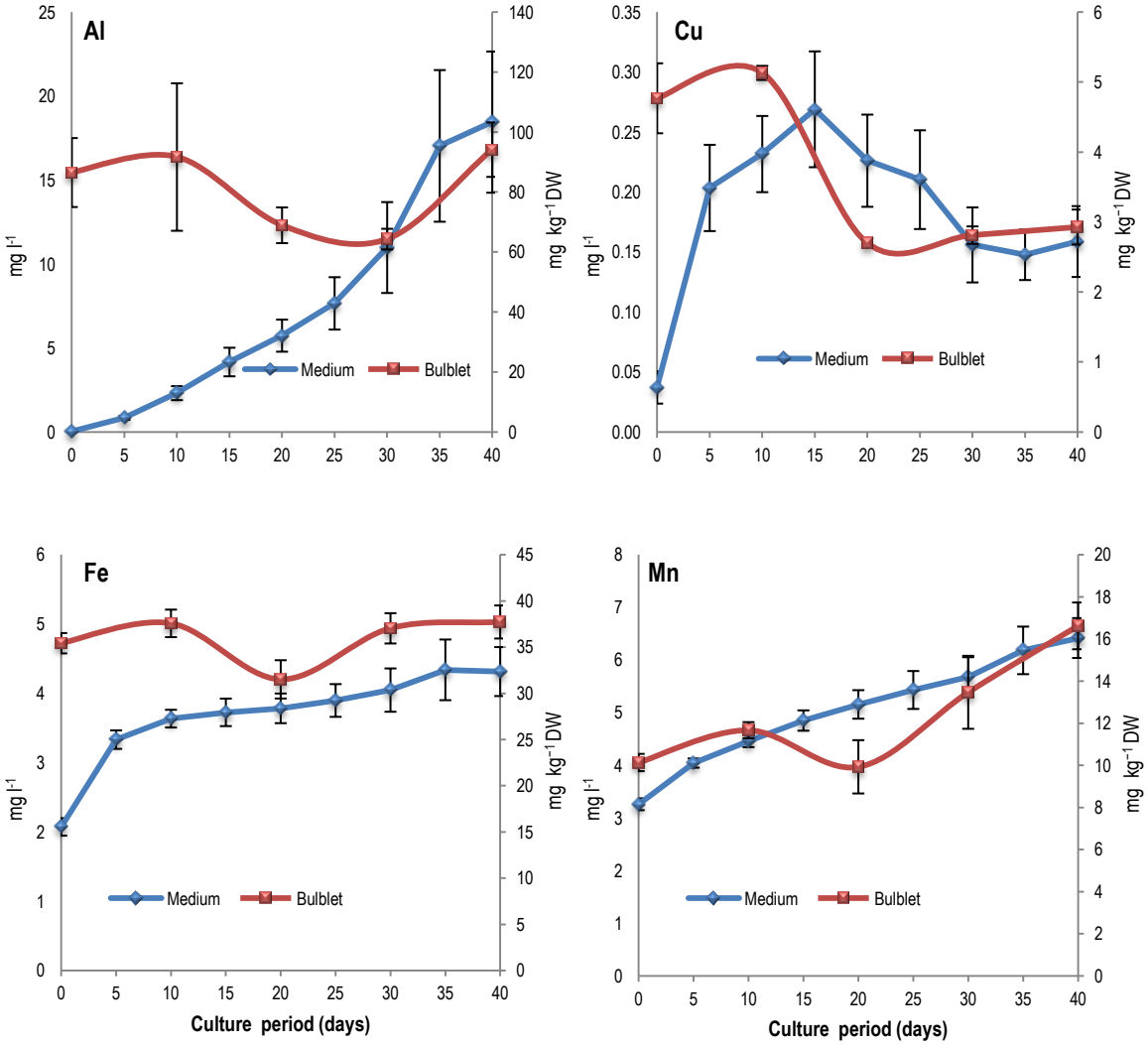


Fig 7. Time-course changes on Macro-and micro-nutrients composition in bulblets and nutrient medium during bioreactor culture of *Lilium* (LA) hybrid "Brindisi"

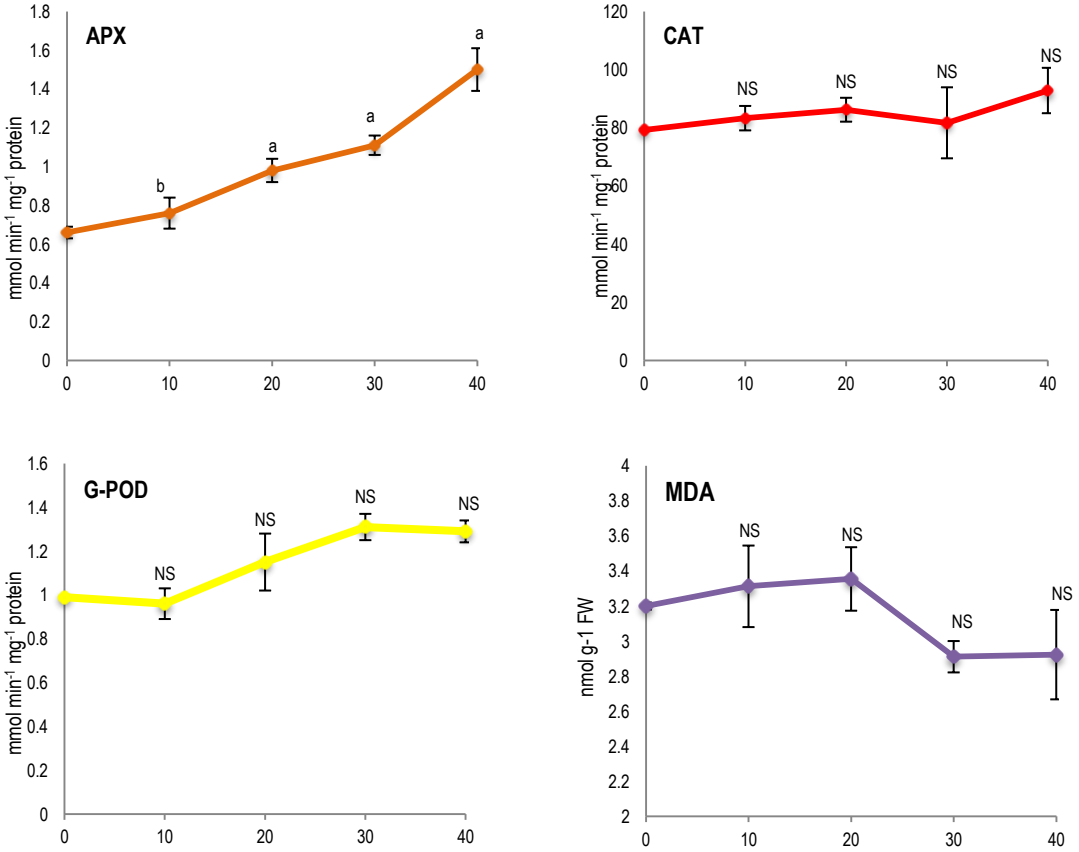
Dynamic on stress levels, antioxidant properties and* chlorophyll pigments *in bioreactor culture of Lilium

The activity of CAT, G-POD and the lipid peroxidation MDA content in bulblets did not shown a significant difference during the whole culture period. However the activity of APX and FRAP, were significantly higher in bulblets from 20 to 40 days of culture (Fig. 8). In addition the production of H_2O_2 increased from initial culture until 40 days. These results indicate that bioreactor culture of *Lilium* induced a low oxidative stress in bulblets as indicated by an enhanced of levels of APX, FRAP and H2O2 probably due to the O_3 exposure of 5 min in bioreactor per day, in fact the formation of H_2O_2 is an important phenomenon of signal transduction induced by stress conditions, which changes the content redox agents and the redox state of cells (Jabs et al., 1997) in

adittion osmotic stress in root suspension cultures of *Hypericum perforatum* resulted in high accumulation of H2O2 (Cui et al., 2010b)

Chlorophyll and carotenoid contents, increased from 10 to 40 days of culture, however Chl a b^{-1} ratio did not shown changes during bioreactor culture, (Fig. 9) these results indicate that increased in pigment contents in bulblets might be due to the high increased in biomass accumulation of *Lilium* in bioreactor culture (Fig. 4).

Changes on stress levels, antioxidant properties in bulblets during bioreactor culture of Lilium (LA) hybrid "Brindisi"



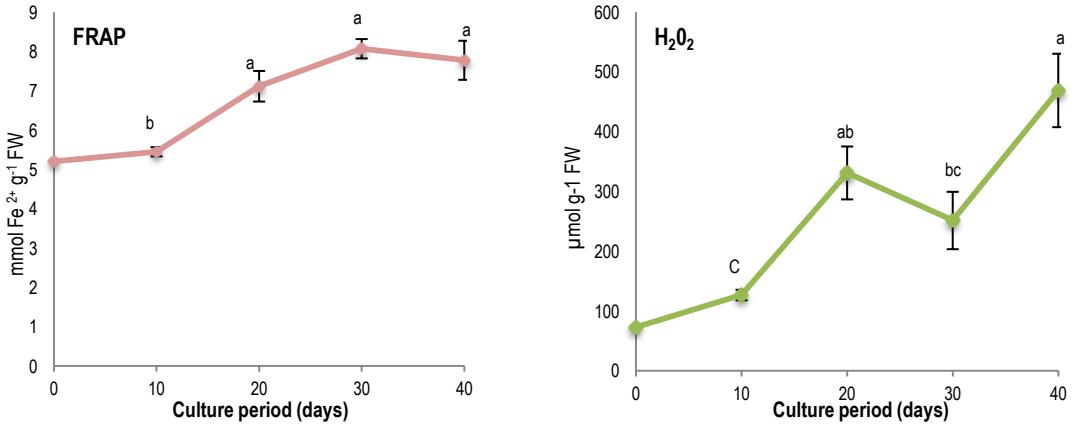


Fig 8. Time-course changes on antioxidant enzyme activities, Lipid peroxidation (MDA), FRAP (ferric reducing-antioxidant power) assay, Hydrogen peroxide contents (H_2O_2)in bulblets during bioreactor culture of *Lilium* (LA) hybrid Brindisi

***Changes* on chlorophyll pigments in bulblets during bioreactor culture of *Lilium* (LA) hybrid "Brindisi "**

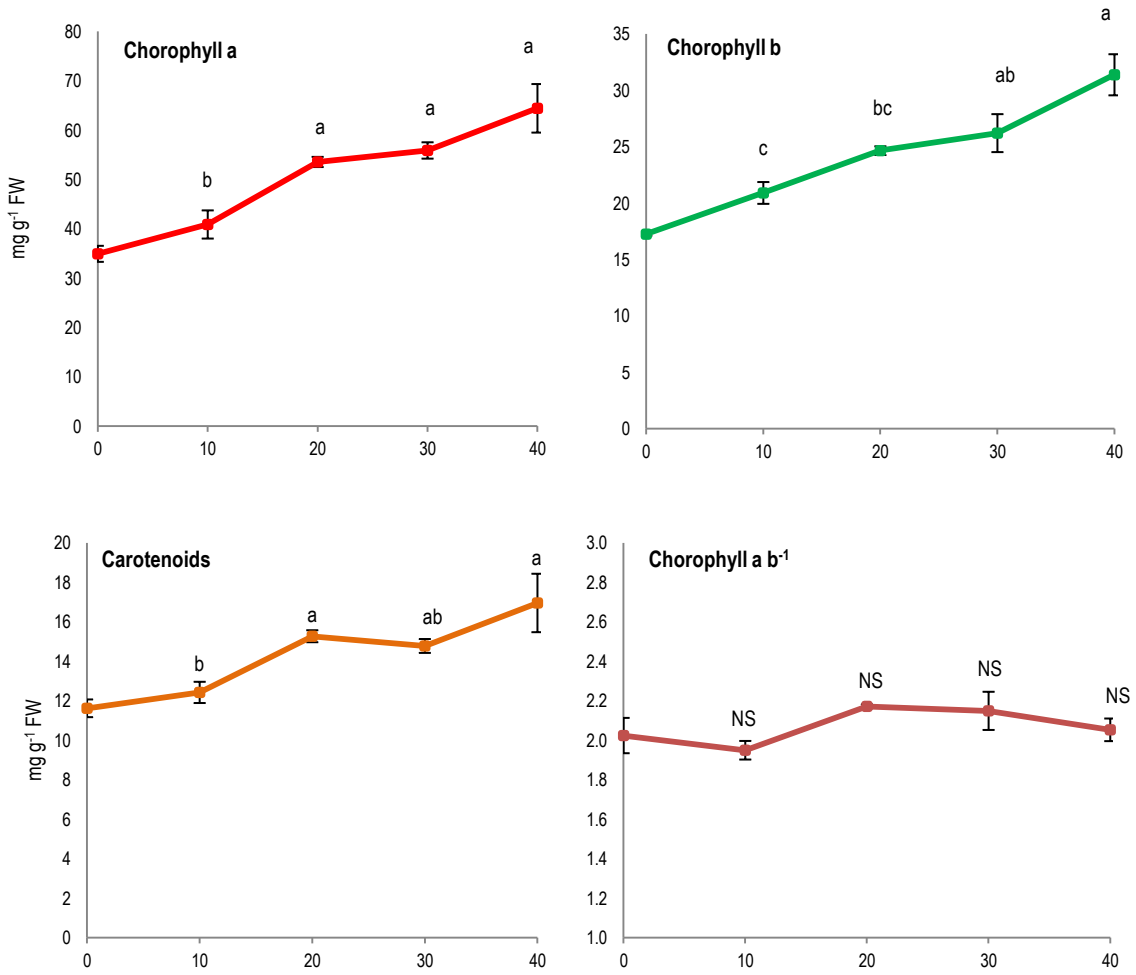


Fig. 9. Time-course changes on chlorophyll pigments in bulblets during bioreactor culture of Lilium (LA) hybrid "Brindisi"

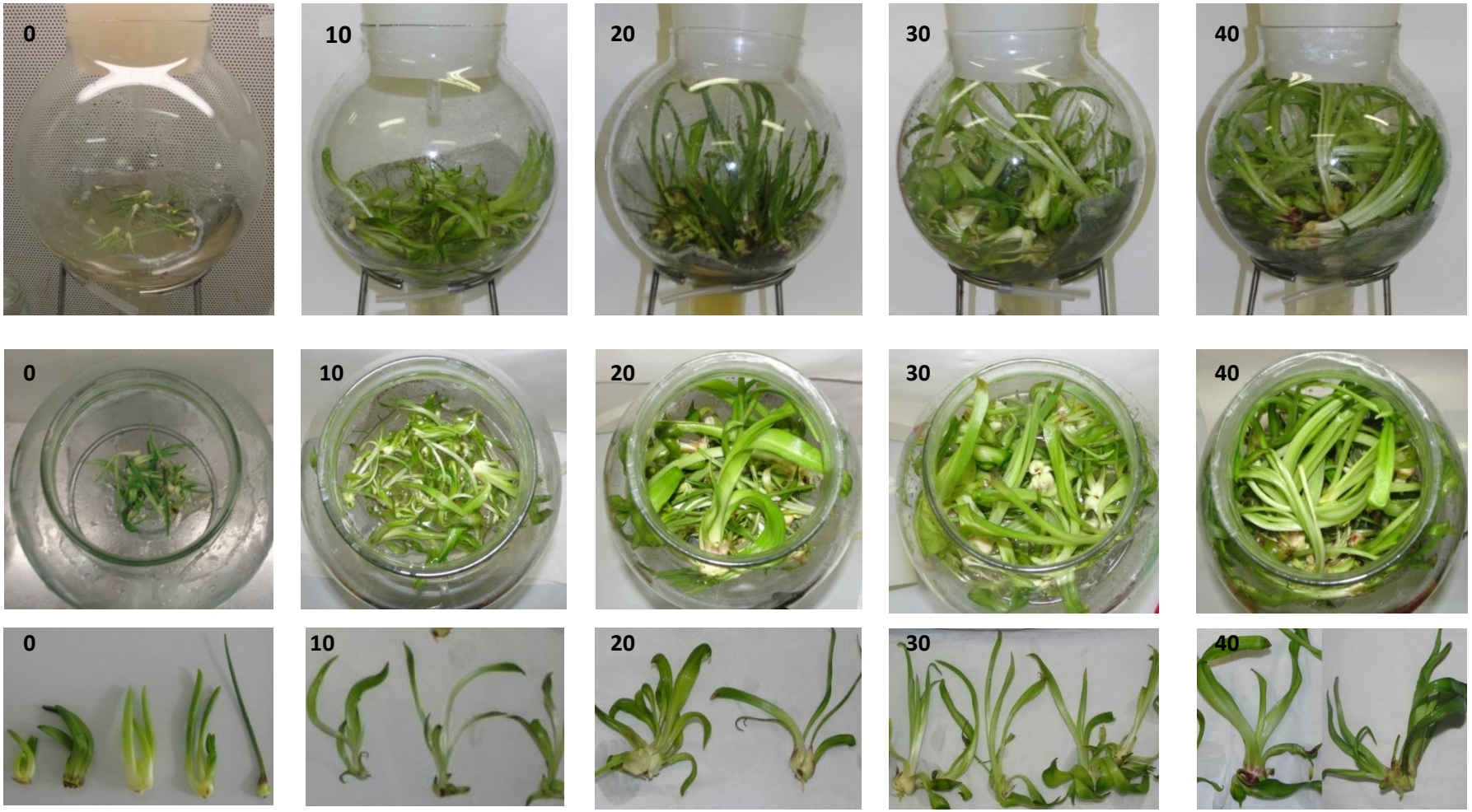


Fig 10. Changes on Bulblet growth during bioreactor culture of *Lilium* (LA) hybrid "Brindisi" The numbers 0, 10, 20, 30 and 40 indicated the culture period in days.

2.4.6 REFERENCES

Barberini S, Savona M, Ruffoni B (2011) Temporary Immersion culture of Lilium bulbiferum. Acta Hortic. 900: 377-379.

Chakrabarty D, Dewir YH, Hahn EJ, Datta SK, Paek KY (2007) The dynamics of nutrient utilization and growth of apple root stock 'M9 EMLA' in temporary versus continuous immersion bioreactors. Plant Growth Regul. 51: 11–19

Cardona Suárez CM, Colla G, Cardarelli M (2011) Response to in vitro propagation of different Asiatic hybrids lilies. Acta Hortic. 900: 393-400

Cardona Suárez C.M., Colla G., Cardarelli, M (2012) Micropropagazione del Lilium in bioreattore: vantaggi e criticità del sistema. Acta Italus Hortus. 6: 119-121.

Cataldo DA, Haroon M, Schrader LE, Young VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6:71–80

Cui XH, Chakrabarty D, Lee EJ, Paek KY (2010a) Production of adventitious roots and secondary metabolites by Hypericum perforatum L. in a bioreactor. Bioresource Technology 101: 4708–4716

Cui XH, Murthy HN, Wu CH, Paek KY (2010b) Adventitious root suspension cultures of Hypericum perforatum: effect of nitrogen source on production of biomass and secondary metabolites. In Vitro Cell.Dev.Biol. Plant 46:437–444

Dantas AK, Majada JP, Fernández B, Cañal MJ (2001) Mineral nutrition in carnation tissue cultures under different ventilation conditions. Plant Growth Regulation. 33:237-243

D' Hasse D, Vandermeiren K, Asard H, Horemans N (2005) Other factors than apoplastic ascorbate contribute to the differential ozone tolerance of two clones of Trifolium repens L. Plant Cell Env. 28: 623-632

Dickson RE, Coleman MD, Pechter P (2001) Growth and crown architecture of two aspen genotypes exposed to interacting ozone and carbon dioxide. Environ Pollut 115:319–334

Estes BL, Enebak SA, Chappalka AH (2000) Loblolly pine seedling growth after inoculation with plant growth promoting rhizobacteria and ozone exposure. Can J For Res 34:1410–1416

Gaucher C, Costanzo N, Afif D (2003) The impact of elevated ozone and carbon dioxide on young Acer saccharum seedlings. Physiol Plant 117:392–402

Gerrits MM, De Klerk GJ (1992) Dry-matter partitioning between bulb and leaves in plantlets of Lilium speciosum regenerated in vitro. Acta Bot. Neerl. 41: 461–468

Gupta P, Sharma AK, Charturvedi HC (1979) Multiplication of Lilium longiflorum Thunb. by asceptic cultures of bulb-scales and their segments. Indian J. Exp. Biol. 16, 940–942

Han BH., Yu, HJ, Yae B.W. Peak K.Y (2004) In vitro micropropagation of Lilium longiflorum 'Georgia'by shoot formation as influenced by addition of liquid medium. J. Hort. Sci. 103: 39–49

Ho CW, Jian WT, Lai HC (2006) Plant regeneration via somatic embryogenesis from suspension cell cultures of Lilium x formolongi hort. using a bioreactor system. In Vitro Cell. Dev. Biol. Plant 42:240–246.

Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor stimulated ion fluxes and O2 − from the oxidative burst are essential components in triggering defense gene activation and phytolexin synthesis in parsley. Proc. Natl. Acad. Sci. 94: 4800– 4805.

Kim YS, Hahn EJ, Paek KY (2001) A large scale production of Lilium bulblets through bioreactor culture. Acta Hort. 560: 383–386.

Leea EJ, Paek KY (2012) Enhanced productivity of biomass and bioactive compounds through bioreactor cultures of Eleutherococcus koreanum Nakai adventitious roots affected by medium salt strength. Industrial Crops and Products 36: 460–465

Li MX, He XY, Chen W, Zhang HL (2007) Effects of elevated CO2 and/or O3 on hormone IAA in needles of Chinese pine. Plant Growth Regul. 53:25–31

Lian ML, Chakrabarty D, Paek KY (2002) Growth and uptake of sucrose and mineral ions by bulblets of Lilium Oriental Hybrid 'Casablanca' during bioreactor culture. J. Hortic. Sci. Biotechnol. 77: 253–257

Lian ML, Chakrabarty D, Paek, KY (2003a) Growth of Lilium Oriental Hybrid 'Casablanca' bulblet using bioreactor culture. Scientia Hort. 97: 41-48

Lian ML, Chakrabarty D, Paek KY (2003b) Bulblet formation from bulbscale segments of Lilium using bioreactor system. Biologia Plantarum 46: 199-203

Liebl RA, Zehr UB., Teyker RH (1992) Influence of nitrogen form on extracellular pH and bentazon uptake by cultured soybean (Glycine max) cells. Weed Sci. 40, 418–423

Lim S, Seon JH, Paek KY, Son SH (1998) Development of pilot scale process for mass production of Lilium bulblets in vitro. In: Drew RA (ed) Proc. Int. Symp. Biotechnology Tropical and Subtropical Species (pp. 237–241) Acta Horti

Luna C, Collavino, M, Sansberro P, Mroginski L (2009) Bacterial contamination in Ilex dumosa (aquifoliaceae) cultures: antibiotic treatment. Acta Hort. (ISHS) 812:97-102.

Low PS, Merida JR (1996) The oxidative burst in plant defense: function and signal transduction. Physiol Plant 96:533–542

Maene L, Debergh P (1985) Liquid medium additions to established tissue cultures to improve elongation and rooting in vivo. Plant Cell Tiss. Org. Cult. 5: 23–33

Nightingale AE (1979) Bulblet formation on Lilium longiflorum Thunb. Nellie White by foliar spray applications of PBA. HortScience 14: 67–68

Novak FJ, Petru E (1981) Tissue propagation of Lilium hybrids. Sci. Hort. 14: 191-199

Seon JH, Kim YS, Son SH, Paek KY (2000) The fed-batch culture system using bioreactor for the bulblet production of oriental lilies. Acta Hort. 520:53-59

Sergiev I, Alexieva V, Karanov E (1997) Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. Compt Rend Acad Bulg Sci 51:121–124

Simonton W, Robacker C, Krueger S (1991) A programmable micropropagation apparatus using cycled medium. Plant Cell Tiss. Org. Cult. 27: 211–218

Shin KS, Chakrabarty D, Ko JY, Han SS, Paek KY (2003) Sucrose utilization and mineral nutrient uptake during hairy root growth of red beet (Beta vulgaris L.) in liquid culture. Plant Growth Regulation 39: 187–193

Skärby L, Ro-Poulsen H, Wellburn FAM, Sheppard LJ (1998) Impacts of ozone on forests: a European perspective. – New Phytol. 139: 109-122

Sluis CJ, Walker KA (1985) Commercialization of plant tissue culture propagation. Intl. Assoc. Plant Tiss. Cult. Newsl. 47: 2– 12

Stimart, DP, Ascher PD (1978) Tissue culture of bulb scale sections for asexual propagation of Lilium longiflorum

Stimart DP, Ascher PD (1981) Foliar emergence from bulblets of Lilium longiflorum Thunb. as related to in vitro regeneration temperatures. J. Am. Soc. Hort. Sci. 106: 446–450

Sunkar R, Bartels D, Kirch HH (2003) Overexpresion of a stress inducibile aldehyde dehydrogenasa gene from Arabidiposis thaliana in transgenic plants improves stress tolerance. Plant J. 35: 452-464

Tanimoto S, Matsubara Y (1995) Stimulating effect of spermine on bulblet formation in bulb-scale segments of Lilium longiflorum. Plant Cell Rep. 15, 297–300

Priyadarshi S, Sen S (1992) A revised scheme for mass propagation of Easter Lily. Plant Cell Tissue Organ Cult. 30:193–197

Takayama S, Misawa M (1980) Differentiation in Lilium bulb scale grown in vitro: effects of activated charcoal. Physiol. Plant. 48, 121–125. Thunb. J. Am. Soc. Hort. Sci. 103, 182–184

Takayama S, Misawa M (1983) A scheme for mass propagation of Lilium in vitro. Sci. Hort. 18: 353–362

Takayama S, Misawa M (1983) The mass propagation of Lilium in vitro by stimulation of multiple adventitious bulb-scale formation and by shake culture. Can. J. Bot. 61: 224-228

Takahasshi S, Matsubara K, Yamagata H, Morimoto, T (1992). Micropropagation of virus free bulblets of Lilium longiflorum by tank culture. 1. Development, culture method and large scale propagation. Acta Hort. 319: 83-88

Tsumaki N &Takayama S (1992) Simple mass propagation of Lilium auratum by shake and jar fermentor culture. J. Soc. High Technol. Agric. 3: 124-128

Van Tuyl, JM, Arens P (2011) Lilium: breeding history of the modern cultivar assortment. Acta Hort. (ISHS) 900:223-230

Yang J.F, Piao XC, Sun D, Lian ML (2010) Production-of-protocorm-like-bodies-with-bioreactor-and-regeneration-in-vitro-of-Oncidium-Sugar-Sweet Scientia Hort 125: 712-717