Regulating Gene Expression in High-scale Plants Micropropagation

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ABSTRACT

Signaling mechanisms have been elicited towards the priming and biopriming stages during micropropagation in Temporary Immersion Bioreactors (TIBs) in sugarcane as model plant. CO₂-rich TIBs induce a photosynthetic condition adequate for the production of natural phenolic metabolites, altogether increasing multiplication rates and functional plants rooting. When combined with Gluconacetobacter diazotrophicus inoculations during transplanting, originate a significant improvement of the percentage of plant adaptability to natural conditions. A more efficient micropropagation process has been optimized on the basis of an accurate exploitation of the natural plant physiology.

Key words: Gene expression, priming, biopriming, TIBs, sugarcane

INTRODUCTION

Plant tissue culture refers to growing and multiplication of cells, tissues and organs on defined solid or liquid media under aseptic and controlled environment. Forty years of in vitro plant research has delivered many well-developed systems that are routinely applied to scientific and commercial activities, namely: (a) micropropagation of genotypes; (b) production of disease-free material from excised apical meristems; (c) international germplasm exchange; (d) generation of somaclones; (e) rapid disease and pest resistance screening; and (f) germplasm conservation (Snyman et al., 2011).

Plant micropropagation procedures are conducted under conditions as natural or similar to those in which the plants will be ultimately grown ex vitro (Ahloowalia et al., 2004). The individual plant species, varieties and clones require specific modification of the growth media, weaning and hardening conditions. In general, the process of plant micropropagation is universally divided into well-defined stages depending on genotype/specie (Debnath, 2011).

Network discovery is a generic term describing the effort of elucidating the nature of relationships between molecules and associated properties emerging from of a biological network. Multiple types of networks have been described with respect to the types of molecules involved and the dimension of the molecular network (Weitz et al., 2007). Similar efforts are under way to construct plant transcriptional regulatory networks, for example those that control flower and root development (Grieneisen et al., 2007), photomorphogenesis (Jiao et al., 2007; Nemhauser, 2008) or the circadian clock (Zeilinger et al., 2006).
Technological advances in biological experimentation are now enabling researchers to investigate living systems on an unprecedented scale by studying genomes, proteomes or molecular networks in their entirety. Whether gene expression analysis provide a rich source of quantitative biological information that allows researchers to move beyond a reductionist approach by both integrating and understanding interactions between multiple components in cells, organisms and processes (Baginsky et al., 2010).

This study considers plant micropropagation as a complex physiological network where each step must believe as an independent one, i.e., establishment, multiplication, rooting, adaptation to field conditions, etc. Integration/management of gene expression systems in parts of the entire micropropagation process (network) have been conducted in sugarcane as model plant. Regulations of gene expression towards useful traits increase the productive efficiency during plant micropropagation integrating both basic and applied researches.

**Temporary Immersion Bioreactors (TIBs):** Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. A more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and pH than the conventional culture vessels is providing by bioreactors increasing the multiplication rates and growth of plant cultures (Levin and Tanny, 2004; Paek et al., 2005). The environment of the growth room determines the light, temperature and gases in the bioreactor vessels (Morini and Melai, 2004).

In temporary immersion bioreactors, the cultures are immersed in the medium, for a preset duration at specified intervals in dependence of plant species and genotypes (Adelberg and Simpson, 2002; Debnath, 2011). TIBs demonstrate a positive effect on plant physiology regulating key pathways as photosynthesis, respiration, transport and nutrient assimilation. As consequences both cell development and cell division rates are significantly enhanced (Etienne and Berthouly, 2002). Environmental factors influencing plant physiology, including the presence of systemic microorganisms (benefic, pathogenic and contaminants), during plant micropropagation are show in Fig. 1.

The influence of these environmental factors on plant physiology could be considered similar to those for conventional micropropagation. Nevertheless, key differences with the time and quality

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Fig. 1: Factors influencing plant micropropagation in Temporary Immersion Bioreactors
of the exposure are demonstrated throughout the interactions. Other significant difference is the improved availability to manipulate or regulate, the interactions using automatic devices. For superior practical use of bioreactor cultures, research aimed at improving the physical and chemical environments such as increased air exchange, increased Photosynthetic Photon Flux (PPF) and optimized CO₂ content are necessary (Paek et al., 2001; Morini and Melai, 2004).

Prevention of contamination in bioreactors requires a proper handling of the plant material, of equipment during transfers and cultures during production. Culture contamination which is a major problem in conventional commercial micropropagation, is even more acute in TIBs (Leifert, 2000). Only the surface sterilized explants, indexed as disease-free must be used to initiate cultures in bioreactors. However, despite the precautions taken in initiating cultures, bioreactors can become contaminated from the environmental microbes. It is noticeable that photomixotrophic plant cultures show a significant diminution or even control of environmental contamination which could be also associated with the significant reduction of sucrose in the culture medium and the production of natural bioactive phytochemicals (Sivakumar, 2006; Arencibia et al., 2008).

Considering the main advantages and disadvantages, the use of bioreactors has led to the development of suitable technologies for plant propagation. Currently, various plant species as sugarcane, pineapple, banana, between others, are propagated using bioreactors to certified seeds production (Escalona et al., 1999, 2003; Ibaraki and Kurata, 2001; Etienne and Berthouly, 2002; Chakrabarty et al., 2003; Arencibia et al., 2008).

**Priming:** One of the important plant adaptations to complex environment challenges is priming behavior (Conrath et al., 2006; Beckers and Conrath, 2007; Frost et al., 2008). In principle, priming means that plants that previously experienced abiotic or biotic stress have altered and most often enhanced their ability to resist and survive recurring stress conditions. In the current terminology, ‘priming’ is usually associated with biotic stresses while ‘hardening’ is used for response adaptations of plants to abiotic factors (Bruce et al., 2007). Priming effects span trophic levels: plants can be primed by herbivore attack, pathogen infection and colonization with micro-organisms, exposure to the metabolites these organisms produce and even synthetic compounds (Conrath et al., 2001).

Conrath et al. (2001) proposed that the accumulation of signaling proteins in their inactive form and their rapid activation in new stress situations can contribute to the formation of short-term stress imprints. Protein phosphorylation and dephosphorylation is one of the most important reversible post-translational modifications that causes inactive proteins to become active and vice versa (Van Bentem and Hirt, 2007). Members of a diverse class of Mitogen Activated Protein Kinases (MAPK) are known to play important roles in mediating pathogen resistance as well as in JA-dependent signal transduction cascades (Soo et al., 2007; Takahashi et al., 2007; Wu et al., 2007; Iriti et al., 2007). A concrete example in Arabidopsis shows that the priming competence induced by treatment with salicylic acid analog benzo (1,2,3) thiadiazole-7-carboxylic acid S-methyl ester (BTH), sold under trade name BionTM (Syngenta AG, Basel, Switzerland) and used commercially for priming and protecting crops against plant pathogens (Iriti et al., 2007), can be attributed to the accumulation of inactive MAPK3 proteins (Conrath et al., 2006); these are activated in response to pathogen infection, thereby enhancing the expression of defense genes and the accumulation of antifungal metabolites.

In addition, more permanent but still reversible changes in gene expression, namely those involved in protection from stress have been described in plants. In particular, DNA methylation
(Mathieu et al., 2007), histone modifications and changes in small-RNA (smRNAs) populations play a role in plant defense-related memory mechanisms. Pandey and Baldwin (2008) can lead to heritable or transgenerational alterations in plant behavior, some of which cannot be explained by Mendelian genetics. Readers are directed to examples of several current reviews that analyze the broad areas of DNA and histone modifications in epigenetic control of development and stress responses (Bronner et al., 2007; Boyko and Kovalchuk, 2008).

**Phenolic metabolites as priming molecules:** Sugarcane, a major source of world sugar and bioenergy production (Wadowasky et al., 2010), is growing throughout the tropics and subtropics. In this sense, as all major plant-based industries require extensive breeding programs to produce/introduce improved genotypes (e.g., sugar content) that are matched to specific selection sites, accounting for biotic (e.g., pest and disease resistance) and abiotic factors in each part of the world (Snyman et al., 2011).

The use of temporary immersion systems in sugarcane promoted both phenolic excretion and shoots formation (Lorenzo et al., 2001). In this way, a genomic characterization of plants has been achieved by suppressing key genes of the phenylpropanoid pathway; as a result, a new function of phenolic metabolites as priming molecules has been characterized during sugarcane micropropagation in TIBs. Genes related to cell metabolism and development (10), plant defenses (9), phenylpropanoids (7), methyl jasmonate response (5), ethylene (5), oxidative burst (3) and auxins (3) pathways, among others (8) were found to be induced in sugarcane plants micropropagating in TIBs with phenolic metabolites, supporting that phenylpropanoids might act as elicitor molecules of others biochemical pathways (Arencibia et al., 2008). As conclusion phenolics related to the brown color in the culture medium display a beneficial role for the induction/expression of genes during sugarcane propagated in TIBs.

The priming approach has been integrated into the sugarcane micropropagation technology by TIBs. Sugarcane micropropagation in CO₂-rich TIBs induces a mixotrophic condition adequate for the production of natural phenolic metabolites (Fig. 2a). Scaling up has been conducted in several commercial genotypes. While phenolics demonstrate to act as priming molecules during the in vitro culture, vitroplantlets growing and shooting in the presence of phenolic metabolites display an enhanced vigor measured as plant size (Fig. 2b,c), emitted functional roots and increase adaptability to the natural environment. Additionally, when combined with the inoculation of the endophytic *Gluconacetobacter diazotrophicus*, a significant improvement of the percentage of survival has been attached through this critical step (Arencibia et al., 2008; Bernal et al., 2008).

Sugarcane phenolic metabolites as elicitors of resistance to tomato bacterial wilt in the *Solanum lycopersicum* and *Ralstonia solanacearum* pathosystem has also been identified (Yang et al., 2010). The culture media was collected and the phenolics were sprayed onto tomato plants infected with *R. solanacearum*, eliciting and/or maintaining an early defense signaling mechanism that resulted in the protection of the plant against the tomato bacterial wilt disease. RT-PCR analyses confirmed that selected genes from defense-related pathways were differentially expressed between plants treated with sugarcane metabolites, non-treated pathogen-free plants and non-treated plants inoculated with *R. solanacearum* (Yang et al., 2010). Results indicate a promising potential for diversification of the sugarcane micropropagation industry by the production of useful phenolic metabolites as byproducts.

The phenylpropanoid pathway is an important pathway in secondary plant metabolism, yielding a variety of phenolics with structural and defense related functions. These phenolic
compounds include lignins, phenolic acids, flavonoids and stilbenes. In addition, enzymes such as phenylalanine ammonia lyase (PAL; EC 4.3.1.5), cinnamate-4-hydroxylase (C4H; EC 1.14.13.11) and 4-coumarate:coenzyme A ligase (4CL; EC 6.2.1.12) are considered to be crucial to phenylpropanoid metabolism. A number of reports have shown that phenylpropanoid derivatives are capable of protecting plants against various biotic (infection by viruses, bacteria, fungi) and abiotic (low and high temperatures, UV-B light, wounding) stresses (Sgarbi et al., 2003; Solecka and Kacperska, 2003). Stilbene synthase (STS) (EC 2.3.1.95) catalyses the last step of the phenylpropanoid biosynthesis pathway leading to the formation of stilbene phytoalexins. Expression of STS genes is often induced in response to biotic and abiotic stresses (Jeandet et al., 2002).

Beside, BABA (beta aminobutyric acid) a non-protein amino acid, was used to induce resistance in grapevine against downy mildew (Slaughter et al., 2008). BABA-induced resistance was observed in the susceptible cv. Chasselas as well as in the resistant cv. Solaris. Following BABA treatment, sporulation of Plasmopara viticola was strongly reduced and the accumulation of stilbenes increased with time following infection. Furthermore, BABA-treatment of Solaris led to a rapid increase in transcript levels of three genes involved in the phenylpropanoid pathway: phenylalanine ammonia lyase, cinnamate-4-hydroxylase and stilbene synthase. BABA-primed Chasselas showed increased transcript levels for cinnamate-4-hydroxylase and stilbene synthase. As a result, the susceptible cultivar became more resistant to downy mildew. In parallel, in grapevine, it has been shown that callose deposition as well as defense mechanisms depending on the phenylpropanoid and the Jasmonic Acid (JA) pathways all contributed to BABA-IR (Hamiduzzaman et al., 2005).

**Bioprimer**: Many symbiotic organisms contribute to the vigor and ability of the tissue-cultured plants to perform well in the field. In the course of tissue culture, plants are made free from such beneficial microbes, e.g., symbiotic nitrogen fixing endophytic bacteria and mycorrhizae. There is no practical way to retain the beneficial microorganisms during tissue culture. The deliberate re-infection of propagules with selected strains can be a valid way of retrieving the benefits of such microbes as a bioprimering approach.

Endophytic microorganisms can promote plant growth, as well as suppress diseases. Plant growth promotion is taken to result from improved nutrient acquisition or hormonal stimulation (Suman et al., 2005). Disease suppression can occur through induction of resistance in the plant (Arencibia et al., 2006).

Several bacterial strains have been shown to act as plant growth-promoting bacteria (PGPR) through both stimulation of growth and Induced Systemic Resistance (ISR) but it is not clear in how far both mechanisms are connected. Induced resistance is manifested as a reduction of the number of diseased plants or in disease severity upon subsequent infection by a pathogen. Such reduced disease susceptibility can be local or systemic, result from developmental or environmental factors and depend on multiple mechanisms (Van Loon, 2007).

The spectrum of diseases to which PGPR elicited ISR confers enhanced resistance overlaps partly with that of pathogen-induced Systemic Acquired Resistance (SAR). Although ISR-eliciting bacteria can induce typical early defense-related responses in cell suspensions, in plants they do not necessarily activate defense-related gene expression. Instead, they appear to act through priming of effective resistance mechanisms, as reflected by earlier and stronger defense reactions once infection occurs (Van Loon, 2007).
While, these bacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites. Several of these metabolites can act as signaling compounds that are perceived by neighboring cells within the same micro-colony, by cells of other bacteria that are present or by cells of the host plant (Van Loon and Bakker, 2003; Bais et al., 2004; Gray and Smith, 2005; Kiely et al., 2006).

The specificity in the reactions of different plant species to individual strains indicates that the reactions of plants to resistance-inducing PGPR must be the outcome of a dynamic interplay between the production and the perception of ISR eliciting signals. Whereas, some PGPR activate defence-related gene expression, others appear to act solely through priming of effective resistance mechanisms, as reflected by earlier and stronger defence reactions once infection occurs (Gray and Smith, 2005; Kiely et al., 2006).

**Gluconacetobacter diazotrophicus as bioprimer:** *Gluconacetobacter diazotrophicus, Herbaspirillum sp., Azospirillum amazonense, Burkholderia spp.,* capable of fixing nitrogen have been reported to colonize the epidermis of sugarcane stem and roots, of which *Gluconacetobacter* (earlier *Acetobacter diazotrophicus*) seems to contribute substantially to nitrogen nutrition of the plant (Dobereiner et al., 1993; James et al., 2001). *Gluconacetobacter diazotrophicus,* a nitrogen fixing endophyte, is found in high number in all part of sugarcane and its better colonization in sugarcane is probably due to its capability to grow in the presence of high sugar and low pH (Dobereiner et al., 1993).

Production of plant growth hormones is the other beneficial trait associated with *Gluconacetobacter diazotrophicus* (Bastin et al., 1998; James et al., 2001). The exact role of such endophytic colonization individually or in a complex endophytic community is not yet very clear but inoculation experiments involving micropropagated plants suggest the positive colonization and its contribution to plant growth and development in terms of improved plant height, nitrogenase activity, leaf nitrogen, biomass and yield (Dobereiner et al., 1993; Sevilla et al., 2001; Muthukumarasamy et al., 2002).

More recently, a new role for the plant growth-promoting nitrogen-fixing endophytic bacteria *Gluconacetobacter diazotrophicus* has been identified and characterized while it is involved in the sugarcane-*Xanthomonas albilineans* pathogenic interactions. Living *G. diazotrophicus* possess and/or produce elicitor molecules which activate the sugarcane defense response resulting in the plant resistance to *X. albilineans,* in this particular case controlling the pathogen transmission to emerging aseptic shoots. A total of 47 differentially expressed transcript derived fragments (TDFs) were identified by cDNA-AFLP. Transcripts showed significant homologies to genes of the ethylene signaling pathway (26%), proteins regulates by auxins (9%), β-1,3 Glucanase proteins (6%) and ubiquitin genes (4%), all major signaling mechanisms. Results point toward a form of induction of systemic resistance in sugarcane, *G. diazotrophicus* interactions which protect the plant against *X. albilineans* attack (Arencibia et al., 2006).

Bioprimer approach has also been integrated into the sugarcane micropropagation technology by Temporary Immersion Bioreactors (TIBs). While phenolics demonstrate to enhance plant capability to be colonized by the endophytic *Gluconacetobacter diazotrophicus,* a simple procedure for *G. diazotrophicus* inoculation has been developed using sugarcane vitrplants during transplanting (Fig. 2d, e). As result a significant improvement of the percentage of adaptability and plant growth rate have been demonstrated in high scale plants micropropagation (Bernal et al., 2008).
Fig. 2(a-f): Large-scale sugarcane micropropagation in (a) CO₂-rich TIBs, (b) induction of a functional rooting during the last in vitro step as an evidence of the photomixotrophic stage, (c-d) sugarcane vitroplantlets obtained in a TIB using 5 L of the culture medium, (e) inoculation of vitroplantlets with a suspension of G. diazotrophicus and planting in a soil-compost-zeolite mixture and (f) twenty-days olds sugarcane plants adapted to the natural environment.

Besides sugarcane, Gluonacetobacter diazotrophicus colonizes many other sugar and non-sugar plants like Pennisetum purpureum, Ipomea batatas Döbereiner, Coffea arabica (Jimenez-Salgado et al., 1997), Eleusine coracana (Loganathan et al., 1999) and Ananas comosus (Tapia-Hernandez et al., 2000). Present strategy could be applied to other plants from which recently Gluonacetobacter diazotrophicus has also been isolated (Cocking et al., 2005), i.e., Arabidopsis thaliana and the crop plants maize (Zea mays), rice (Oryza sativa), wheat (Triticum aestivum), oilseed rape (Brassica napus), tomato (Lycopersicon esculentum) and white clover (Trifolium repens).

For a bioinoculant to benefit the plant, it should establish and compete with the native heterotrophic bacterial population and also should acclimatize the local conditions. Under such conditions the indigenous bioinoculant strains may perform better than introduced alien ones for promoting plant growth due to their superior adaptability to the environment. Therefore, selection of superior strains of Gluonacetobacter diazotrophicus is essential for its exploitation as bioinoculant for improving growth of in vitro sugarcane plants (Oliveira et al., 2002).

As summary views of the sugarcane micropropagation process is show in Fig. 2.

An assessment between the conventional micropropagation technology (agar base) and the new one including CO₂-rich TIBs producing phenolics and a further inoculation with G. diazotrophicus is show in Table 1. The crucial steps influencing the main parameters related to productive efficiency are under layer considering the conventional micropropagation as the comparison base.
Table 1: Comparing high-scale plant micropropagation technologies in sugarcane as model plant

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<th>Conventional</th>
<th>TIBs</th>
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<tr>
<td>Multiplication rates</td>
<td>1: 3-5</td>
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<td></td>
<td>1: 10-15</td>
<td>1: 80-120</td>
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<td>Steps (handwork)</td>
<td>Initiation</td>
<td>Initiation</td>
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<td></td>
<td>10 multiplication subcultures</td>
<td>3 multiplication subcultures</td>
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<td></td>
<td>rooting</td>
<td>TIBs multiplication</td>
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<td>Complete cycle</td>
<td>12 months including 10 multiplication subcultures</td>
<td>5 months including 3 multiplication subcultures</td>
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<tr>
<td>Expected production in 5 months starting from 10 explants</td>
<td>3000 units</td>
<td>20000 units</td>
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<tr>
<td>In vitro consumables and cultures medium to produce 1 million plants</td>
<td>100%</td>
<td>30%</td>
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<tr>
<td>Time of laminar flow operations to produce 1 million plants (handwork)</td>
<td>100%</td>
<td>30%</td>
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<tr>
<td>Total laboratory area to produce 1 million units</td>
<td>100%</td>
<td>40%</td>
</tr>
<tr>
<td>Cost per unit</td>
<td>100%</td>
<td>25-30%</td>
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Remarks:

- The optimization for photomixotrophic conditions is required in a case by case manner using the TIBs as a plant multiplication platform. A permanent goal must be reach the whole autotrophic stage at least in the final in vitro stage.
- Wherever possible, the priming and biopriming strategies should be standardized according to local conditions, as well as considering the specific plant-microbe interactions.
- The integration of new approaches for plant micropogmentation should result in a more efficient and competitive technology, where both basic and applied researches must be primarily focused to take profit of the natural plant physiology during the in vitro stage.

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REFERENCES


