IN VITRO REGENERATION AND PROLIFERATION OF *SPATHIPHYLLUM* HYBRIDS THROUGH A SPECIAL WAY

PhD thesis

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1. PRELIMINARIES AND AIMS OF THE STUDY

My thesis deals with the *in vitro* propagation of *Spathiphyllum floribundum* cv. 'Petite' using a new regeneration method which has not been used before. The method is based on a bud-like special organ form (GGb – green globular bud) which is formed *in vivo* on its own, or can be formed *in vitro* from floral spadices using plant growth regulators. The new regeneration and proliferation method uses organogenesis which provides high genetic stability and at the same time it renders large-scale multiplication in bioreactors possible. The aims of my experiments are to study:

- the induction and development of these organ forms from floral spadices and shoots,
- their applicability to multiplication in plant tissue culture,
- the conditions and the process of shoot regeneration from GGb
- the possibility of using this form and regeneration method as an alternative to shoot cultures.

Above all I strive to examine the phenomenon from a practical point of view hoping that my results will be applicable more easily and directly in the practice. Since the literature does not refer to such or similar phenomenon, the subject is quite new. Therefore I use empirical approach to study the topic considering my available possibilities.

2. MATERIAL AND METHOD

2.1 Initiating sterile cultures

Previously established sterile cultures (both GGb and shoot cultures) were at my disposal in the laboratory therefore I focused on the induction of GGb form during sterile culture initiation. I tried to start the sterile cultures with many kind of explant types: blooming floral spadices (in different phenophases), vegetative buds or spadices from already acclimatized plants. To sum up I performed 5 initiating experiments with 3 cultivars using different sterilization methods and media.

2.2 Experiments with the GGb form

2.2.1 Evaluation of different NAA concentration

To study the GGb-to-shoot regeneration I combined the NAA in low concentrations (0, 0.5, 0.75, 1, 1.25, 1.5 mgL^{-1}) with 0.25 mgL^{-1} BA. The base of the media was ½× MS, supplemented with 20 gL^{-1} sucrose. The experiment went on 6 months. At the end I evaluated the diameter of GGb clusters, ranked their state, counted the number of GGbs in a cluster. I measured the length of shoots and roots and counted their amount in the clusters where shoot regeneration could be observed.

2.2.2 Application of different cytokinins in liquid media

To study the suitability of GGb form to liquid culture I examined their reaction in media without any kind of solidifying agent. I supplemented the liquid media with three kind of cytokinins: BA, BR, MT and with a cytokinin-like compound: TDZ, each in four concentrations (0.05, 0.1, 0.2, 0.5 mgL^{-1}). I evaluated the GGb clusters after 3 months in each treatment.

The diameter, weight and peroxidase activity of GGb clusters were measured, the number of GGbs in a cluster was counted and I ranked the clusters according to their developmental state.

2.2.3 Effect of raised macroelement and sucrose concentration

On solidified medium in the presence of 0.5 mgL^{-1} BA and 0.1 mgL^{-1} NAA I examined the effect of macroelement and sucrose concentration, whether they have any effect on the develeopment of GGb clusters. 1× and 2× MS macroelement concentration were combined with 20 or 40 gL^{-1} sucrose, as a control I used 0,5× MS macroelement levels without sucrose. After 2 month I measured the morphological characteristics of the GGb clusters. Biochemical parameters were also determined: the chlorophyll and carotenoid content and the peroxidase (POD) activity of the clusters.

2.2.4 Effect of triazole type growth retardants

With the use of 0.5 mgL^{-1} paclobutrazol (PBZ) I achieved high rate shoot regeneration from GGbs, therefore I examined the progress of the regeneration and characterized with the changes in POD activity levels. Then I evaluated the usable concentration interval of PBZ with a wide concentration range (0.25, 0.5, 1, 2 mgL^{-1}), without any kind of growth regulators on agar solidifed ½× MS medium with 20 gL^{-1} sucrose. I measured the state of GGb clusters after 8 and 12 weeks and measured the POD activity.

The examination of flurprimidol (FP) happened in four concentrations (0.05 mgL^{-1}, 0.1 mgL^{-1}, 0.2 mgL^{-1} and 0.5 mgL^{-1}) along with 0.2 mgL^{-1} BA and 0.1 mgL^{-1} NAA on solidified ½× MS media with 20 gL^{-1} sucrose. After 3 month I determined the developmental stage of the GGb clusters, their

morphological characteristics and POD activity levels. To study the post-treatment effect of FP, living or regenerating GGb clusters were passed onto ½× MS medium with 0.5 mgL^{-1} BA and 0.1 mgL^{-1} NAA supplemented with 20 gL^{-1} sucrose. After 5 months samples were taken from the GGb clusters in different stages of regeneration to measure the POD activity levels.

2.3 Experiments with the shoot cultures of *Spathiphyllum floribundum*

The experiments with the shoot cultures had two main aims: to decide if GGb form can also be induced from shoots and to optimize the conditions further in shoot cultures to achieve better multiplication via organogenesis – authors in literature only deal with growth regulators, they do not mention the use of different macroelement concentration and carbohydrate sources.

2.3.1 Effect of different carbohydrate sources

Carbohydrates of 4 different types – two disaccharides (saccharose, maltose) and two monosaccharides (glucose and fructose) were evaluated as an energy source for shoot cultures. These sugars were used in a concentration of 20 gL^{-1} in solidified MS media supplemented with 0.5 mgL^{-1} BA and 0.1 mgL^{-1} NAA. The following parameters of the plant samples were measured: weight gain, shoot and leaf number, shoot height and increase in leaf surface. Net photosynthetic rate (CO_2-assimilation rate), stomatal conductivity and transpiration were determined as well.

2.3.2 Effect of raised macroelement and sucrose concentration

This experiment lasted 4 months and I evaluated two concentrations of sucrose and fructose (20 and 40 gL^{-1}) combined with ½×, 1× and 2× MS macroelement concentrations to study the effect of these factors on the long-term culturing of the shoots without change of the medium.

Morphological characteristics, dry weight percentage, chlorophyll-content and POD activity were measured at the evaluation.

2.3.3 Effect of paclobutrazol

The growth retarding agent paclobutrazol was also evaluated in shoot cultures to find out if it can cause shoot dwarving to such extent which results in GGb form. Beside the PBZ-free control medium I made 4 treatments with 0.25, 0.5, 1 and 2 mgL^{-1} PBZ. Plants were kept on these media for 100 days then morphological evaluation was followed (shoot number, shoot height, root number, weight of shoot clusters), chlorophyll-content, POD activity and gas exchange parameters were also determined. After another 50 days on growth regulator free medium I determined morphological characteristics, POD activity and gas exchange to study the the post-treatment effect of PBZ. Plants left untouched after the measurements were acclimatised.

2.4 Measurement of morphological parameters

2.4.1 GGb clusters

The morphological characteristics of the GGb clusters were determined under steril environment in laminar air flow hood. I measured the diameter of the clusters, number of GGbs in each cluster, the number of regenerated shoots (if any) , and roots. To characterize the form of the clusters, I used a form index which uses the following formula: $|x-y|/\max\{x,y\}$, where x and y are the width and length of the clusters.

Developmental stages of GGb clusters were ranked according to a subjective scale (Fig. 1.).

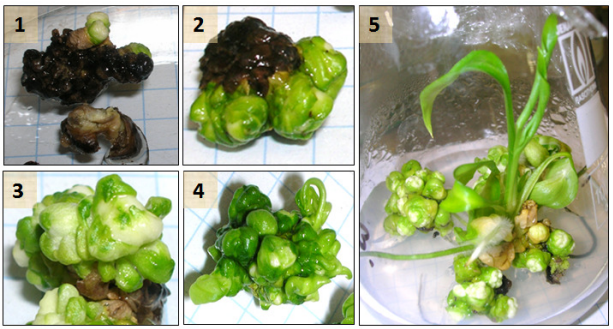

Fig. 1. The different developmental stages of GGb clusters in *Spathiphyllum floribundum* 'Petite' *in vitro* cultures.
1-dead, 2-half dead, 3-living, 4-shoot regeneration in progress, 5-at least one shoot regeneration is complete

2.4.2 Shoot cultures

I measured the number of shoots, leaves and roots, the height of the shoots. To measure the leaf surface I scanned them at 600 dpi resolution after removing them individually from the plants. To determine the form index of the leaves the same formula was used like at the GGb clusters.

2.5 Measurement of gas-exchange parameters

The gas exchange intensity was measured with a portable infrared gas analysator. During the measurements a so-called 'conifer chamber' was used into which a whole plant could be placed. Measurements were carried out in the second third of the light period. Transpiration rate, CO_2 stomatal conductivity and net photosynthetic rate of the plants were measured immediately after opening the vessels.

2.6 Anatomical studies

For studying the state of stomatal pores, a nitrocellulose based colorless varnish was applied to the surface of the fully expanded two leaves of the

shoot tips creating a negative facial replica. Half-thin (30 µm) cross sections were made from the leaves using a microtome. Replicas and dissections were examined under phase-contrast without staining, measurements were made with AxioVision LE 4.8.2.0.

2.7 Measurement of biochemical parameters

The chlorophyll and carotenoid content were measured spectrophotometrically using the method of Arnon (1949).

To measure the dry weight content, the plant material was dried at 80 °C for 24 hours until constant weight.

Peroxidase (EC 1.11.1.7) (POD) activity in the leaf tissues were measured also spectrophotometrically (λ = 460 nm) in the presence of H_2O_2 as substrate and ortodianisidine (ε = 11,3) as chromogen reagent after Shannon et al. (1966).

3. RESULTS

3.1 Initiation of sterile cultures

An effective sterilization procedure was successfully elaborated during the sterile culture initiation attempts. The third attempt was the first successful one during which all of the explants turned green and swelled one and a half months after inoculation but did not show the signs of regeneration to shoots. Regenerating dwarf shoots appeared on one of the spadix explants in the fifth month, these primordia resembled a GGb form which just has been started to regenerate into shoot. After separating and placing these shoots on fresh medium containing cytokinin I noticed the developing of normal GGb forms (Fig. 2.).

Fig. 2. Induction of GGbs on tissues separated from the floral spadix of a *Spathiphyllum* hybrid on a medium with 3 mgL^{-1} MT

In the fourth and fifth initiation attempt I used the explants of the cultivar 'Petite' and an other cv. of *S. floribundum* (not specified closer). Antibiotic treatment was omitted from the sterilization procedure and the explants were placed on media containing 3 mgL^{-1} MT. Regeneration could not be observed on the explants of the unknown cultivar but on the explants of

‘Petite’ GGb forms started to develop distinctly one month after inoculation. These GGbs started to grow afterwards (Fig. 3.).

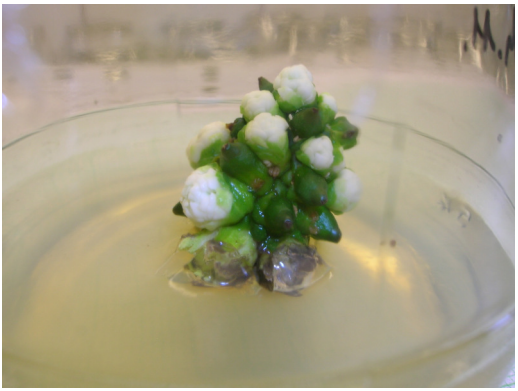

Fig. 3. Developing GGbs on the floral spadix of *Spathiphyllum floribundum* ‘Petite' after 2 months of initiation on a medium with 3 mgL^{-1} MT

The fourth initiation attempt using the cultivar ‘Petite’ resulted GGb development in 6 cases from the 13 explants each in two months. The fifth attempt resulted one successful GGb development from 15 cases.

3.2 Experiments with the GGb clusters of *Spathiphyllum*

3.2.1 Evaluation of different NAA concentrations

I found that different NAA levels did not changed significantly the diameter of GGb clusters nor the individual size and number of GGbs inside a cluster. The growing pace of GGb clusters is the same in each NAA treatment but the shoot regeneration rate from GGbs is different: the lowest NAA concentration (0.5 mgL^{-1}) caused highest GGb-to-shoot regeneration (22.6%). The NAA level of 0.75 mgL^{-1} is ideal to maintain the GGb clusters

in the GGb form, this medium kept the majority (83.3%) of the clusters unchanged.

3.2.2 Evaluating different cytokinins in liquid media

The increase of GGb number inside a cluster characterizes the multiplication of GGb cluster the best. This value was the highest by high levels of BA and TDZ in the media (Fig. 4.).

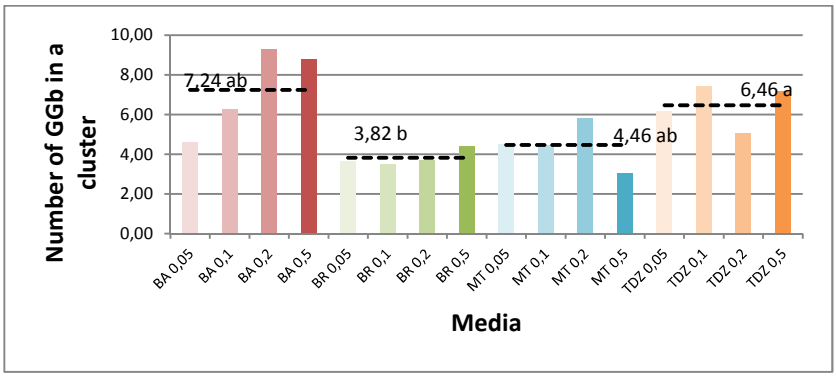

Fig. 4. Values of GGb number in a cluster by different cytokinin type and concentration in *in vitro* cultures of *Spathiphyllum floribundum* 'Petite' using liquid media

BR containing media did not increase the starter GGb number (3-5 pcs/cluster) significantly just like MT treatments. But the diameter of the clusters were increased by MT better than by BR, this means that MT increases not the number but the size of the individual GGBs in a cluster. In accordance with this fact the specific weight of the GGBs is also the highest by MT treatments. Liquid media with cytokinins did not cause GGb-to-shoot regeneration in the most cases.

3.2.3 Evaluation of increased macroelement and saccharose concentration

Solid media containing saccharose kept the GGb clusters unchanged: at least 85% of them did not show shoot regeneration. Doubling the macroelement levels in the media increased this percentage even higher above 95%. In the presence of saccharose GGb clusters were fully vital but the lack of sucrose caused 41% of the GGb clusters to deteriorate. The GGb-to-shoot regeneration is inhibited by high sucrose or macroelement concentration (Fig. 5.).

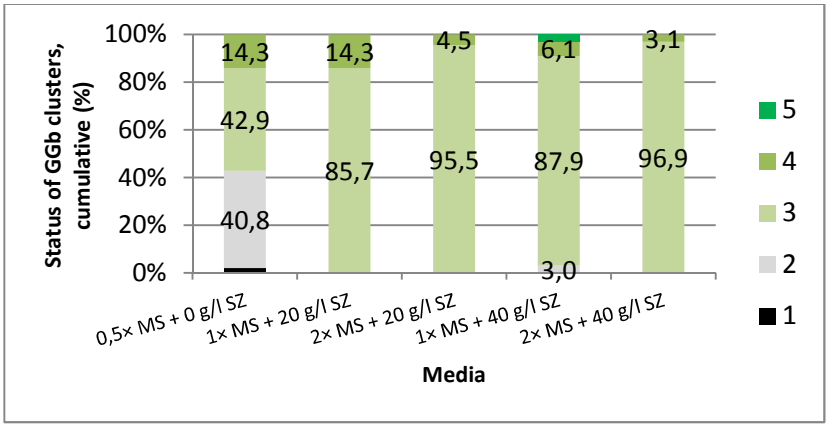

Fig. 5. Effect of increased macroelement and sucrose concentration on the status of GGb clusters in *in vitro* cultures of *Spathiphyllum floribundum* 'Petite'. 1-dead, 2-half dead, 3-living, 4-shoot regeneration in progress, 5-at least one shoot regeneration is complete

The peroxidase activity of GGb clusters (Fig. 6.) show an increase with the estimated osmolality ($\psi_{sum}=\psi_{elements}+\psi_{sucrose}+\psi_{agar}$) of the medium, thus the more higher is the solubilized dry weight content of the medium the more higher is the POD activity in the GGb clusters due to the abiotic stress.

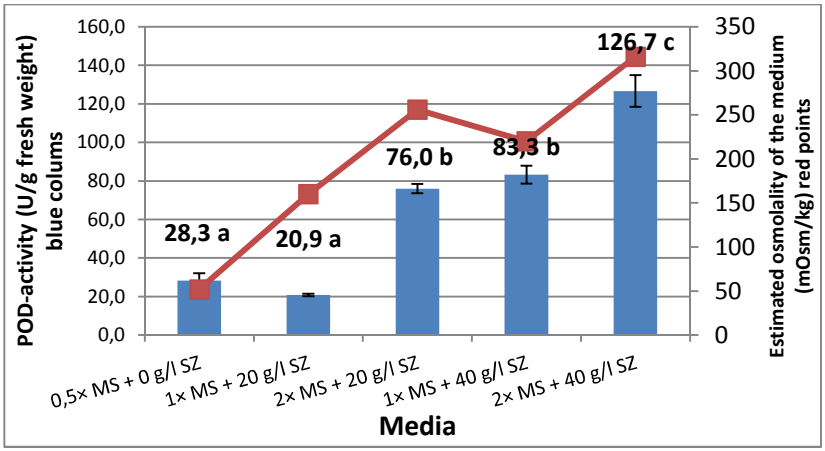

Fig. 6. The effect of raised macroelement and sucrose concentration in connection with the osmolality of the medium on POD activity of the GGbs in the *in vitro* cultures of *Spathiphyllum floribundum* 'Petite'.

3.2.4 Evaluating the effect of triazole type growth retardants

The process of GGb-to-shoot regeneration could be observed in the medium containing 0.5 mgL^{-1} paclobutrazol (PBZ) and 0.2 mgL^{-1} BA, different stages of regenerating GGb clusters were present at the same time. Examining these stages the following grouping describes the process the best (Fig. 7.):

Fig. 7. Stages of GGb-to-shoot regeneration in *in vitro* cultures of *Spathiphyllum floribundum* 'Petite'

0. The GGb is bud-like, the green leaf primordia covering the side of the GGb body with a white shoot tip protruding out of them. This is the normal GGb stage before regeneration.
1. The leaf primordia are larger and fully covering the white tip like a cabbage.
2. The tip is opening, white leaf primordium emerging from the white part.
3. The uppermost white leaf primordium starts to become green.
4. The leaf primordium continues to grow and start to unfold.
5. The leaf blade is already visible but the petiole is quite short yet.
6. The petiole expands and the shoot axis become also visible.

Root development is typical in the sixth phase but it can occur rarely earlier. I found during biochemical examinations that the activity of peroxidase enzymes characterizes quite well the different stages of GGb-to-shoot regeneration process (Fig. 8.).

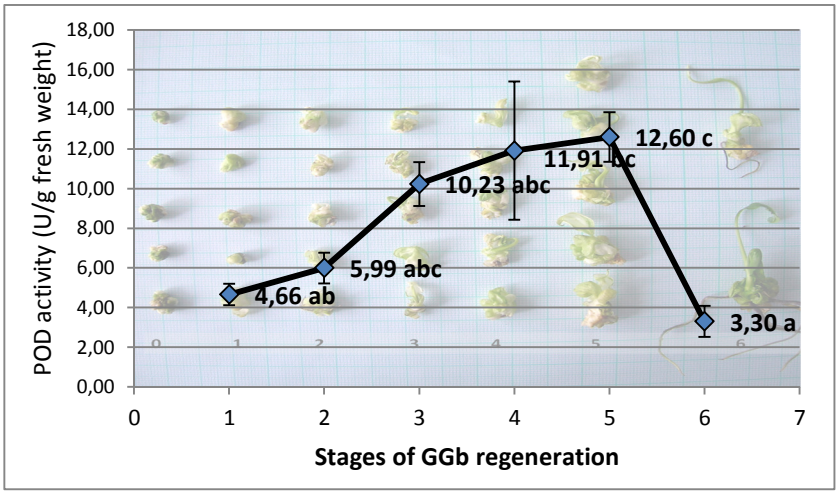

Fig. 8. The values of POD activity in the different regeneration stages of GGb in cultures of *Spathiphyllum floribundum* 'Petite'

The POD activity rises as the regeneration progresses and falls down quickly at the end. PBZ causes lower POD acitivity in GGb clusters than media without PBZ.

The usable concentration of PBZ to GGbs was tested without any other growth regulators on half strength MS medium with 20 gL^{-1} saccharose. Examined concentrations were: 0.25; 0.5; 1; 2 mgL^{-1}. The highest PBZ level caused the GGb clusters to deteriorate completely 10 days after inoculation. 2 mgL^{-1} of PBZ is toxic to GGbs.

The concentrations of flurprimidol (FP) were selected in a smaller range according to the results of the PBZ experiment and the media also contained 0.2 mgL^{-1} BA and 0.1 mgL^{-1} NAA. Each concentration of FP caused some GGb deterioration examining their state in the 12th week of experiment, but the least degree was at 0.1 mgL^{-1}. The ratio of shoot regenerating GGbs reached 26% by this concentration. Number of GGbs in a cluster was about 8 in average after 8 weeks which corresponds to the multiplication rate on liquid media and it is higher than that all the other solid media produced. To examine the late effect of FP treatments the GGb clusters were placed on a medium with 0.5 mgL^{-1} BA and 0.1 mgL^{-1} NAA and 5 months after inoculation the ratio of regenerated GGb was evaluated. The late effect of the treatments with 0.1 and 0.2 mgL^{-1} FP caused 50% rate of GGb-to-shoot regeneration 5 months after inoculation on media with 0.5 mgL^{-1} BA.

3.3 Experiments with shoot cultures of *Spathiphyllum*

3.3.1 Evaluating of different carbohydrate types

Net photosynthetic rate is shown in Fig. 9. by the effect of different treatments. It is clearly visible that the values of CO_2 assimilation rate was positive in every case except one (8th week, fructose treatment, but this treatment also caused positive value in average). This means that the plants absorbed more CO_2 than that was released during their respiration already at the start of their culture.

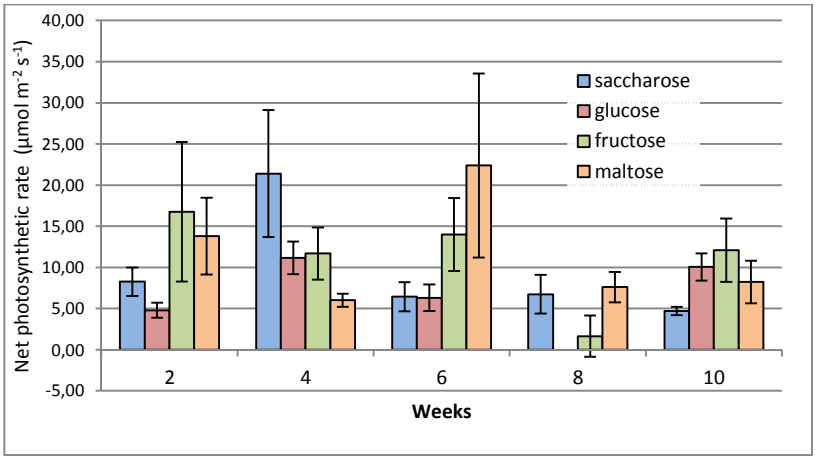

Fig. 9. The effect of different carbohydrate sources on the net photosynthetic rate of *Spathiphyllum floribundum* 'Petite' shoot cultures.

3.3.2 The effect of paclobutrazol

Adventitious shoot formation was not affected by the PBZ treatments compared to the control plants neither during the PBZ treatments nor through the post-treatment period. The average shoot height of all PBZ treated plants was smaller than that of the control plants but PBZ had not such a dwarfing effect in the post-treatment period. In this stage the highest PBZ concentration caused a significant dwarfing effect. Weight values showed a similar trend: PBZ treatments caused a significantly lower weight compared to the control but no dissimilarities were observed among the results of different PBZ concentrations.

Compared to the control, the PBZ treatments did not affect the transpiration and stomatal conductivity but the photosynthetic rate was significantly higher (near four-fold) using 0.25 mg l-1 PBZ. With increasing PBZ concentration, net photosynthetic rate decreases gradually to the control

level. Examining the long-term effects of PBZ revealed that the transpiration and stomatal conductivity also showed significant differences between the treatments. In addition to the still existing differences concerning the net photosynthetic rates – each PBZ treatment caused higher CO_2 assimilation rate compared to the control - the most outstanding treatment was the one with 0.5 mgL^{-1} PBZ; 1 and 2 mgL^{-1} caused significantly lower values. Post-treatment effect on transpiration rates showed a gradual decline with the increasing PBZ concentration. (Fig. 10.).

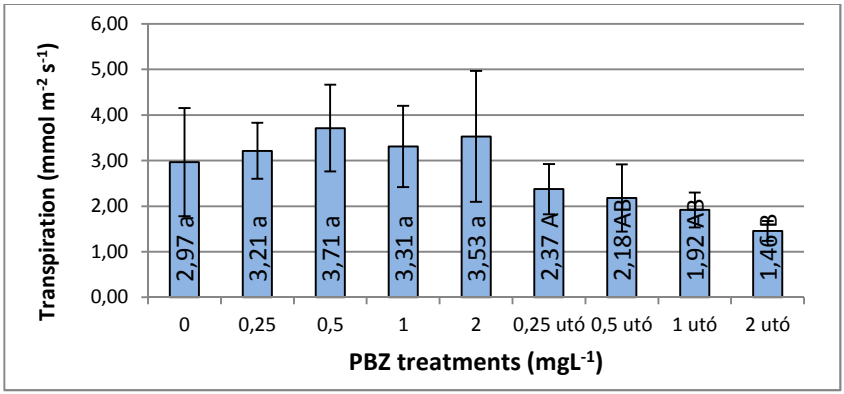

Fig. 10. The transpiration rate in different PBZ treatments in *Spathiphyllum floribundum* 'Petite' shoot cultures

3.3.2.1 Histological analyses

Examination of the surface replicas showed that the stomata pore width and length are notably larger in the 2 mgL^{-1} PBZ treatment than at lower PBZ concentrations or in the control plants, so the stomatal aperture area estimated on the basis of these two parameters were also significantly higher compared to other treatments. The sizes of guard cells (width and length) gradually increased with the increasing PBZ concentrations (Fig. 11.).

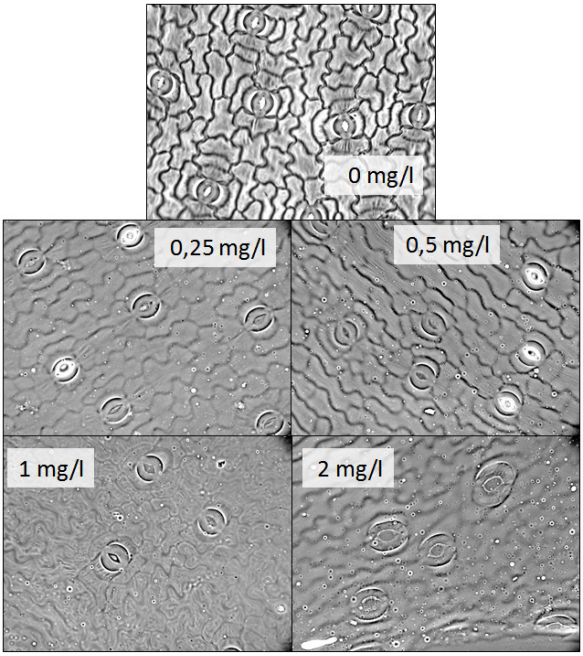

Fig. 11. The size of stomatal pores in the post PBZ treatment period in *Spathiphyllum floribundum* 'Petite' shoot cultures

Examining the cross-sections declared that the leaf thickness was the largest in untreated plants and increasing PBZ concentrations caused gradually thinner leaves. In untreated plants and after the treatment with the lowest PBZ concentration the cells are rounded compared to the plants of high level PBZ exposure. The rate of intercellular spaces of the mesophyll were gradually reduced with increasing PBZ concentrations. Vascular components were found to be more developed after PBZ treatments compared to the control plants. The structure of the mesophyll showed differences, as well: untreated plants had a one-layered palisade parenchyma, the two lowest PBZ concentrations caused the formation of a

two-layered palisade parenchyma, while the cell layers are not discernible at higher PBZ concentrations.

3.4 New scientific results

1) It can be stated that the GGb clusters of *Spathiphyllum floribundum* 'Petite' are suitable for liquid culture systems with continous immersion, and therefore it might be suitable for multiplication in large scale bioreactor systems.
2) It can be stated that the GGb form possess a slower metabolism and growth compared to shoot cultures and therefore it is suitable for longterm gene bank storage with minimal growth technique.
3) It was proved that *in vitro* shoot cultures of *Spathiphyllum floribundum* 'Petite' can be characterized with positive net CO_2 asszimilation rate already at the start of culture despite the presence of carbohydrate sources in the medium.
4) It was determined that the PBZ in a concentration of 2 mgL^{-1} causes widely open stomatal pores, but provokes high turgor and water retention in *in vitro Spathiphyllum floribundum* 'Petite', thus the PBZ induced transpiration decrease is not exclusively bound to the stomatal regulation controlled by abscisic acid.
5) It was declared that there is a large difference between the POD activity levels of the shoot cultures and GGb cluster of *Spathiphyllum floribundum* 'Petite', the GGb form have higher POD-activity than that of the shoots. The examined triazole compounds (paclobutrazol and flurprimidol) are able to decrease efficiently the POD activity of GGb clusters and they alter their developmental way.

6) It was proved that the growth regulating effect of examined triazole compounds not only manifest in growth retardation but applied along with cytokinins they can promote the shoot regeneration from GGb clusters in *Spathiphyllum floribundum* 'Petite'.

7) The GGb form can be described as a dwarf shootprimordium stucked in generative phase, in which an endogenous inhibition caused by gibberellins does not allow the regeneration to normal sized shoots. Triazole growth regulators can resolve this inhibiton and promote the GGb clusters to switch back to vegetative phase resulting in shoot regeneration.

4 CONCLUSIONS

4.1 Initiating cultures

The first two culture initiation attempt were unsuccessful because of the insufficient sterilization method. These two attempt did not include the use of mercury(II)-chloride. At the third attempt I used the desinfection protocol described by HEGEDŰS (2005) with some kind of modifications: I used Nystatin instead of benomyl, and I used mercury(II)-chloride as well, so the efficiency of sterilization was greatly enhanced. Although the desinfection was susccessful, the regeneration of the explants was very low. This can allude to two options: either the type or concentration of used cytokinin is inadequate to trigger regeneration or the antibiotic treatment caused growth retardation in the explants. During the repeated attempts I used the same cytokinin type and concentration (3 mgL^{-1} MT) and by omitting the antibiotics from the sterilization process I succeed the achieve regeneration from the explants. Thus the use of antibiotics and their phytotoxic effects might be responsible for unsuccessful regeneration from the explants. Since the desinfection protocol was effective without the antibiotics as well, using them is not recommended in the culture initiation of *Spathiphyllum* hybrids but the use of mercury(II)-chloride is necessary.

4.2 The GGb form

4.2.1 Multiplication rate of GGb clusters

To compare the proliferation speed of GGb clusters in the different experiments I calculated their average multiplication rate per 2 months. For multiplication the ½× MS medium supplemented with BA or TDZ are the most suitable. Compared to solid media, the liquid ones provide more higher multiplication rate using BA as cytokinin. This high multiplication

rate can be achieved on solid media as well if low concentration of BA is combined with flurprimidol. However triazoles are promoting the GGb clusters to regenerate into shoots, so the best proliferation medium which keeps the GGb form is the liquid ½× MS medium with 20 gL^{-1} sucrose, 0.2 – 0.5 mgL^{-1} BA or 0.05 mgL^{-1} TDZ and 0.1 mgL^{-1} NAA.

4.2.2 The GGb-to-shoot regeneration

The experiments revealed the GGb-to-shoot regeneration frequency in different medium as well. Full inhibition of regeneration only occured if gibberellic acid (GA_3) was used which coincide the results of OROSZ (2006), who tried different type of auxins combined with GA_3 and every treatment resulted in inhibition of the regeneration. The regeneration into shoots by the clusters left alone without any interruption occurs in general if the medium does not contain GA_3, but the process is prolonged and does not happen at the same time by each cluster. OROSZ (2006) proposed, that the regeneration could be bound to size limits but I did not experienced such signs, I observed shoot regeneration equally from small and large clusters as well. Without using triazoles the regeneration rate were between 7-36 %, using triazoles much higher rate could be achieved, but it was not synchronously happening.

During the shoot regeneration the POD activity of GGb clusters always decreased to a lower level. Between the POD activity levels of shoot and GGb clusters there is a difference in order of magnitude. The shoot regeneration always happened the same way in each treatment.

4.2.3 Explanation of the GGb form

The formation of GGb clusters always happen in floral spadices in generative phase, especially in *in vitro* cultures, but I also observed their formation on plants *in vivo* without any exogenous effect. From *in vitro* (vegetative phase) shoot cultures I could not observe their development by the effect of examined growth regulators, retardants, raised macroelement levels or different carbohydrate sources. The literature does not mention such phenomenon except the previous publications of my co-workers. Anatomical studies with scanning electronmicroscopy revealed that GGb clusters are bud-like formations, dwarf shoots (SZALVA, 2003). Their regeneration into normal shoots might be endogenously inhibited despite the fact that they are not in dormancy since they grow and proliferate continously under *in vitro* environment.

During the production of *Spathiphyllum* hybrids GA_3-treatment is a recognized method to bring the plants in generative phase, to induce flowering. Not directly the increase of GA_3-level is responsible for flower induction but the bound processes. The increased level of GA_3 causes oxidative stress in the plants, which activate the γ-glutamylcysteine-synthetase, glutathione synthesis increases. The increase in oxidative stress can be measured easily, the flower-induced and flowering plants have higher POD activity than non-flowering plants (DEWIR et al, 2007). According to my measures GGb clusters have with an order of magnitude higher POD activity compared to vegetative shoot cultures. GGb clusters separated from floral spadix explants keeps their GGb form and high POD activity if they are continously interrupted with cutting and placing them on new medium or if they are placed on medium containing gibberellic acid. Media without GA_3 causes shoot regeneration sooner or later if the clusters are not interrupted. The gibberellin biosynthesis inhibitors can efficiently

decrease the POD level of the clusters and they promote the shoot regeneration. Considering these facts the GGb form can be described as a dwarf shootprimordium stucked in generative phase, in which an endogenous inhibition does not allow the regeneration to normal sized shoots. The gibberellins play a role in the induction and maintenance of this endogenous inhibition. Triazole growth regulators which are inhibiting the gibberellin synthesis are promoting the GGb clusters to switch back to vegetative phase which can be seen in enhanced shoot regeneration and the great and obvious decrease of the POD activity.

4.3 Effects of carbohydrates on shoot cultures

It can be stated examining the effect of different carbohydrates on the development of shoot cultures that the use of monosaccharides (fructose and glucose) in 2 w/v% concentrations resulted in better development of plants compared to sucrose, and the use of maltose is not suggestible. No literature was available so far which had examine other carbohydrate than sucrose in *Spathiphyllum* shoot cultures. According to my results other carbohydrate sources than sucrose are also suitable for *in vitro* cultures of *Spathiphyllum* 'Petite', moreover they have better effects. However the non-reducing sugar, maltose is not recommended. According to my measurements the net CO_2-asszimilation rate values of the plants on media with 20 gL^{-1} (2 %) sucrose are larger with an order of magnitude than that of with 30 gL^{-1} (3 %) or 60 gL^{-1} (6 %) sucrose in *in vitro* cultures of *Spathiphyllum* 'Petite' (results by VAN HUYLENBROECK et DEBERGH, 1996). The higher and positive net CO_2-assimilation rate means also easier acclimatisation (KUBOTA, 2001).

Examining the multiplication rates it can be stated that the results of my experiments and those described in literature are not directly comparable, because not only different carbohydrate levels but also other growth regulator concentrations were used; most frequently 1-2 mgL^{-1} (~4-8 μM) BA was used in literature, while I used smaller, 0.5 mgL^{-1} concentration. It is also hard to standardize the multiplication rate applied to different time periods since I showed that some growth characteristics is not changing in a linear way in the function of time. The most frequently used 1-2 mgL^{-1} BA concentration causes more smaller plants than the concentration of 0.5 mgL^{-1} applied by me. The less rejuvenilized plants need less time to acclimatize thus considering only the absolute amount of newly developed shoots a multiplication technology can not be judged. The other characteristics of these shoots (height, weight – which is not detailed in many papers) influence greatly the required time after the multiplication phase to reach the final acclimatised stage.

4.4 Effect of paclobutrazol on shoot development

PBZ enhances the biosynthesis of endogenous cytokinins (Zhu et al. 2004), however, I have not experienced increase in the shoot number of *Spathiphyllum floribundum.* Nevertheless, growth retardant effect of PBZ manifested obviously in the height of shoots which started to normalize on PBZ-free medium after 50 days. Higher PBZ levels caused significantly lower root and leaf number. As a long-term effect of the highest (2 mgL^{-1}) PBZ concentration the weight of the shoot clumps reached that of the control plants, yet their height did not. This might be due to the elevated assimilate accumulation; however, neither the dry weight content nor the net photosynthetic rate confirmed this hypothesis. The accumulating water quantity may be also responsible for the phenomenon, since it contributes to

the weight gain. In addition, the high turgor of cells causes widely open stomatal pores. The larger stomatal aperture at high PBZ concentrations could be explained by the ability of PBZ to increase endogenous cytokinin (Zhu et al. 2004) and IAA level (Nagy et al. 1991, Zheng et al. 2012), since both cytokinins and particulary IAA promote stomatal opening (Wang et al. 1994, Cousson 2010, Pemadasa 1982). However, I have not found any signs of enhanced cytokinin biosynthesis in the plants, furthermore, the high POD activity measured might be associated with lower IAA levels, because IAA-oxidase features are exclusively bound to peroxidases (Shinshi and Noguchi 1975). Similarly to other triazoles, PBZ can increase the level of abscisic acid in some species (Asare-Boamah et al. 1986, Upreti et al. 2013) but in some cases it can also decrease its amount (Buta et Spaulding 1991, Wang et al. 1987). Abscisic acid plays a principal role in stomatal closure (Liotenberg et al. 1999) and thus in the reduction of transpiration, as well. However, not all *in vitro* plants are responding to abscisic acid with stomatal closure (Brainerd et Fuchigami 1982, Wardle and Short 1983), because of the anatomical aberration of *in vitro* developed stomata (Zeiger 1983). Although Hazarika et al. (2002) observed that PBZ incorporated in medium caused stomatal closure in *Citrus* plants, I found the opposite in case of *Spathiphyllum*: 2 mgL^{-1} PBZ concentration significantly increased the stomatal aperture, at the same time the transpiration rate decreased notably. My results suggest that the PBZ induced transpiration decrease is not exclusively bound to the stomatal regulation controlled by abscisic acid. The explanation for lower transpiration by open stomata could be that the water translocation is obstructed from cells to intercellular spaces, from which the water vapour can get out of the plant tissue passing through the stomata or even through the cuticle. My results showed that high (2 mgL^{-1}) PBZ caused smaller intercellular spaces compared to the other treatments,

and according to Wang et al. (1986) PBZ can change the polysaccharide content of the cell wall — these factors may play a role in water retention. Tari (2003) stated that the transpiration per unit area of PBZ treated bean is not lower than that of non treated plants, and the transpiration decrease by PBZ treatment derives only from smaller leaf surface area. My results indicate that PBZ induced transpiration decrease in *S. floribundum* 'Petite' is also valid in a per unit area sense.

4.5 Practical results

The application of GGb form in the micropropagation of *Spathiphyllum* hybrids can be very prosperous using liquid medium with continous immersion in bioreactor, because there is no need to use support stands to ensure adequate aeration nor ebb-and-flow system as it is required by shoot cultures (WATAD et al., 1997; DEWIR et al., 2006). The GGb form only requires constantly agitated medium to stay alive and to proliferate.

Because of the slower metabolism of GGb clusters compared to shoot cultures this form could be suitable in longterm storage in gene bank for instance using minimal growth techniques.

GGb clusters are viable for months *ex vitro* if humid environment is provided (based on my results not detailed in the study), so if a proper regeneration technology is available, the GGb clusters could be used as a propagating material without the need of acclimatisation as a synthetic seed.

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