A special developmental way in the plant family Araceae

Closure report NKFIH PD 116269

Principal investigator: István Dániel Mosonyi

Institution: Szent István University, Department of Floriculture and Dendrology

Summary

During the research, I examined the appearance of a vegetative bud formulation (hereinafter: bud clusters) on the inflorescences of 16 taxa of 7 genera in the family of *Araceae*. Previously this formulation was observed only on a peace lily variety. No formation was observed in taxa outside the genus of peace lily, but it was found in other varieties of *Spathiphyllum* as well, so this phenomenon may be characteristic of this genus. According to the histological examinations performed, these bud clusters can be considered as dwarf-stemmed shoot initiations, buds and they develop directly on the axis of the inflorescence stem. They can be kept alive and propagated *in vitro* in both solid and liquid media, separated from the inflorescence. According to enzymological studies, the level of oxidative state measured in bud clusters is larger than that of in the vegetative shoots, and this value corresponds to the level measurable in the inflorescences of the plant, which may be created by the different endogenous growth regulatory environment of the generative state and lasts in the bud clusters until shoot development. Gibberellin biosynthesis inhibitors alter this condition and promote the transformation of bud clusters into shoots. These bud clusters have been shown to be suitable for use as propagating material, from which complete shoots can be produced and grown, and a method based on this could potentially become a new and efficient *in vitro* propagation technology for *Spathiphyllum* hybrids.

Figures, charts, tables contain simplified data without units and scale bars, because results are in publication process.

Acquisition of equipments, plant stocks, conference participation In the first months of the project the planned laboratory equipment required for the histological assessment were acquisited after carrying out the tendering procedures: Thermo Scientific Microm HM 355 automatic rotary microtome (refurbished), Kunz Instruments WD-4 paraffin dispenser, Kunz Instruments HIR-3 histological bath, consumables and accessories. In the second research period purchase of a light microscope and a culture rotator equipment were planned. Because of financial reasons (price offers were twice as high as it was planned) instead of a culture rotator which has the same parameters as of the one already in our possession, a tube roller device (Stuart SRT9D) has been acquired which provide similar culturing possibilities in liquid media using plastic centrifuge tubes with caps. The acquired light microscope is an Euromex iScope IS.1153-Pli biological microscope with plan achromatic inifinity corrected objectives, equipped with a phasecontrast set and digital camera. In the last year of the project our plant growing test chamber used for incubation of plant material for gibberellin activity essays and acclimatization of *in vitro* plant material became defective due to the intensive usage, so I also spent on repairing costs. In parallel to the acquisition of histological lab equipments in the beginning of the project the selection of potential plant taxa has begun considering commercial availability, environmental and space requirements. Finally the purchase was made from multiple sources (November 2016 – January 2017): *Aglaonema commutatum* 'Silver Queen' and 'Maria', *Dieffenbachia* 'Bertina', 'Green Magic', 'Summer Style', *Spathiphyllum* 'Bellini',

’Chopin’, ’Strauss’, ’Cupido Compacto’, *Anthurium* ’Almera Rouge’, ’Mont Blanc’, *Alocasia × amazonica* ’Polly’, *Zamioculcas zamiifolia*. After the purchase the plants were conditioned and adapted – transplantation to larger pots, preventive plant protection treatments, propagation by division were made. The plants which were already at our disposal in larger amounts (*Spathiphyllum* ’Petite’, *Syngonium podophyllum*) were treated in the same way.

During the project scientific literature, reference books were also purchased from the fund, and I also participated in a scientific conference: The 13th International Scientific Conference – Biotechnology and quality of raw materials and foodstuffs (2018.09.17-19., Smolenice, Slovakia) in Smolenice, Slovakia presenting my partial achieved results. Another conference participation was also planned in 2020, but it was cancelled due to the coronavirus epidemic. The project has leftover fund which I would like to use for covering the publication cost of an upcoming article sent for publication.

Details of the research work:

***In vivo* bud cluster induction and the observation of their spontaneous formation**. Flowering *Spathiphyllum* plants were treated with high concentrations of meta-topolin solutions (dipping the spadices into the solution for several seconds repeated 3 times on subsequent days) but bud cluster formation was not observable only the precociously greening of spadices. I could observe spontaneous bud cluster formation in the spadices in *in vivo* environment in two cases without any treatment – at *Spathiphyllum* ’Strauss’ and ’Petite’ cultivars. The ’Strauss’ specimens spadix already contained the bud cluster structure at a very early stage of flowering, during the opening of spathe, but it declined when the spadix turned brown without fruit development. Whereas in ’Petite’ specimen the bud cluster became visible during the greening of spadix, after pollination, between the adjacent developing fruit parts, started to develop and was there until the ripening of the fruits (4 months). When the ripen spadix was falling apart, I placed the bud cluster on peat based substrate where it lived on for 1 month before it died without any further development. Along the observation of own plants, specimens in large *Araceae* collections (ELTE Botanic Garden – Budapest and National Botanic Garden – Vácrátót) were observed as well but no bud cluster were found.

In the second year *in vivo* bud cluster development was observed once in Spathiphyllum ‘Strauss’ cultivar (without cytokinin treatment), this bud cluster was left in the spadix for 2 months to develop until I harvested, fixed and prepared the material for histological examination.

In the third year two of the *in vivo* observed inflorescences in *Spathiphyllum* ‘Petite’ mother plants showed bud cluster development in May, similarly to previous years the *in vivo* bud cluster development was happened in the spring season again.

In the fourth year during the flowering of the mother plants grown in the greenhouse, I again observed the appearance of bud clusters on the inflorescences, in spring 3 inflorescences showed *in vivo* bud cluster in *Spathiphyllum* 'Petite' , in early summer (June) I also observed 3 more bud cluster in the same cultivar, and one case in the ’Strauss’ cultivar.

Considering the data of 4 years, compared to the total number of inflorescences, the proportion of inflorescences which showed bud cluster in the case of 'Petite' cultivars varied from 0 to 5.8% (n=39-104), in the case of 'Strauss' cultivars 0 - 4.7% (n = 32-64). No *in vivo* bud cluster were found on the cultivars ‘Bellini’, ‘Cupido Compacto’ and ‘Chopin’.

For other taxa other than the genus *Spathiphyllum*, no bud cluster formation was observed *in vivo*.

***In vitro* culture initiations.** In the first year *in vitro* cultures were started immediately using spadix explants from those mother plants which were acquisited in flowering state (*Spathiphyllum* and

Anthurium cultivars), and several more times right after as new flowers were developing (altogether 6 times) *Aglaonema* cultivars has started to flower in May 2017 but unfortunately culture starting was possible only one time in June at the end of blooming period because of building reconstruction works in the laboratory. By the other plants which are normally sold without flowers (*Dieffenbachia* cultivars, *Alocasia*, *Zamicoculcas*, *Syngonium*) were treated with gibberellic acid in the spring to induce flowering (and also those *Spathiphyllum* and *Anthurium* cultivars which were flowering in January). *Alocasia × amazonica* was the first to bloom in July 2017, *in vitro* culture iniation was taken place during the summer. *Dieffenbachia* cultivars have entered generative phase at the end of the summer, culture initiation had taken place at that time. The majority of culture initiations were failed due to endogenous bacterial contaminants at those plant bought in flowering state, however successful sterilization and *in vitro* bud cluster development could be observed in one case both in *Spathiphyllum* 'Strauss' and 'Cupido Compacto', as well as in the case of the existing 'Petite' variety, I also experienced the induction of a bud clusters on auxin-dominant medium, contrary to previous experience (previous experience in bud cluster formation were bound to high cytokinin concentration). Successfully disinfected *Anthurium* explants did not show development or regeneration in inducing media used for *Spathiphyllum* cultivars. *Aglaonema* cultivars flowering 4 months after purchase were much easier to disinfect before culture initiation but instead of shoot or bud cluster formation parthenocarp fruit development has taken place just like in the majority of *Spathiphyllum* explants without bud clusters. However bud cluster formation and fruit development are not mutually exclusive as *in vivo* observations has showed.

In the second year due to the lack of success in previous year's experiments to induce bud clusters in *in vivo* environment with cytokinin applications most of the developing spadix inflorescences were used as an explant to start *in vitro* cultures in order to observe the formation of bud clusters. During this research period, I had the opportunity to initiate sterile culture 12 times as I continuously harvested the spadices from the mother plants.

Dieffenbachia taxa were involved in culture initiation 2 times observing the effect of 4 different thidiazuron concentrations, *Aglaonema* taxa were involved 2 times as well using media supplemented with gibberellin-antagonist paclobutrazol instead of gibberellin containing media used last year which did not result in shoot or bud cluster induction, only parthenocarpic fruit establishment were observable. Both in the case of *Dieffenbachia* and *Aglaonema* no bud cluster formation took place as a result of the media used in the experiments. The male parts of the spadix inflorescences showed no signs of development or change at all, they died slowly whereas in the female parts parthenocarpic fruit development started, the developing fruits either if separated from or leaved in the axis of the spadix showed ripening, and after that the tissues were decayed with age but no shoot regeneration or bud cluster development occurred.

In the case of *Alocasia macrorrhiza* culture initiation happened once, the male and female parts of the inflorescences showed the same results as mentioned above in *Dieffenbachia* or *Aglaonema*. The media used for this purpose were the same which caused - even if in just few cases – bud cluster induction in *Spathiphyllum*: benzyl-adenine + naphthalene acetic acid (cytokinin dominant, ratio 30:1), or benzyl-adenin + 2,4-dichlorophenoxy acetic acid (cytokinin dominant, ratio 4:1).

The two *Anthurium* cultivars were involved in culture initiation 3 times using spadices and pieces from leaves, stem and roots as well. The surface sterilization was very difficult in this plant, only 2 spadices were successfully disinfected from the 37. Additional 12 spadices with mild (not aggressively spreading) bacterial infection were kept in culture for further observation. Spadices became green and swelling fruit primordia appeared but no shoot or bud cluster development were present.

4 cultivars of *Spathiphyllum* were used 4 times to induce bud cluster form again on thidiazuron enriched media (4 different concentrations - the same as used in Dieffenbachia). 2 months after initiation all of the successfully surface sterilized spadices decayed in the thidiazuron containing media independently from the concentration or the presence of 2,4-D. Some of the spadices were put in kinetin or meta-topolin containing media but no bud cluster induction happened in these cases. *Zamioculcas*, *Alocasia × amazonica* and *Syngonium* taxa had very few or not a single inflorescence therefore no culture initation was possible.

In the third year the newly developing spadix inflorescences of *Spathiphyllum* were mostly left on the mother plants in order to observe the possible bud cluster formation in *in vivo* environment. Therefore *in vitro* culture initiation was done only once, using 13 inflorescences of *Spathiphyllum* 'Strauss' and 'Petite'. The spathe leaves were already open around these spadices and no bud cluster was visible on them. These explants were placed on medium supplemented with the following growth regulators: benzylaminopurine and indolebutyric acid (auxin dominant, ratio 2:1) Due to the already opened spathe leaves the efficiency of the disinfection was low, and 80% of the explants remained bacterially infected. No shoot or bud cluster formation was observed on the plant parts.

In the fourth year I initiated *in vitro* sterile culture also only once from 5 young inflorescence explant of the 'Bellini' cultivar, which did not contained bud cluster structure at the time of culture initiation but in the second month after the start-up in one case a bud cluster appeared *in vitro* on one of the explant, I detached it and started to propagate.

Summarizing the data of 4 years the bud cluster formula appeared 8 times *in vitro* when morphologically normally developed spadices were placed on *in vitro* medium, out of the 217 initiated spadices, this is 3.7% of the cases. Inducing media included both cytokine-predominant and auxin-predominant media. In half of the cases, bud cluster formation ('Petite' cultivar) was observed with metatopolin and naphthalene acetic acid (cytokinin dominant, ratio 30:1), but also observed in one case with metatopoline + naphthalene acetic acid (cytokinin dominant, ratio 80:1, 'Cupido Compacto' cultivar), in one case at BA + NAA (cytokinin dominant, ratio: 15:1, 'Bellini' cultivar), and also with 2,4-D + BA (auxin dominant, ratio 4:1) and BA + 2,4-D (cytokinin dominant, ratio 10:1, 'Petite' cultivar). The bases of the media in each case were half strength MS supplemented with sucrose. The appearance of bud clusters, even with very different hormone ratios, suggests that they do not develop primarily under the influence of growth regulators, but may already be present in the spadix placed on the medium, and appear in the *in vitro* environment due to the further development of the detached spadix. Bud clusters appearing on *in vivo* plants, even in the absence of growth-regulating treatments, also support this.

Taxa of other genera did not develop a bud cluster *in vivo* or *in vitro*, so it is likely that this phenomenon is characteristic only of *Spathiphyllum* taxa.

Morphological and histological examination of bud clusters. The bud clusters are spherical formations with an average diameter of 3-5 mm, covered with green on the outside and whitish-colored leaf primordia on the top and inside (Figure 1.)

Figure 1. *In vivo* developed bud cluster on the spadix of ‘Petite’ cultivar

Histological examination revealed that the stem axis of the *in vivo* developed clusters is directly connected to the inflorescence stem axis. (Figure 2).

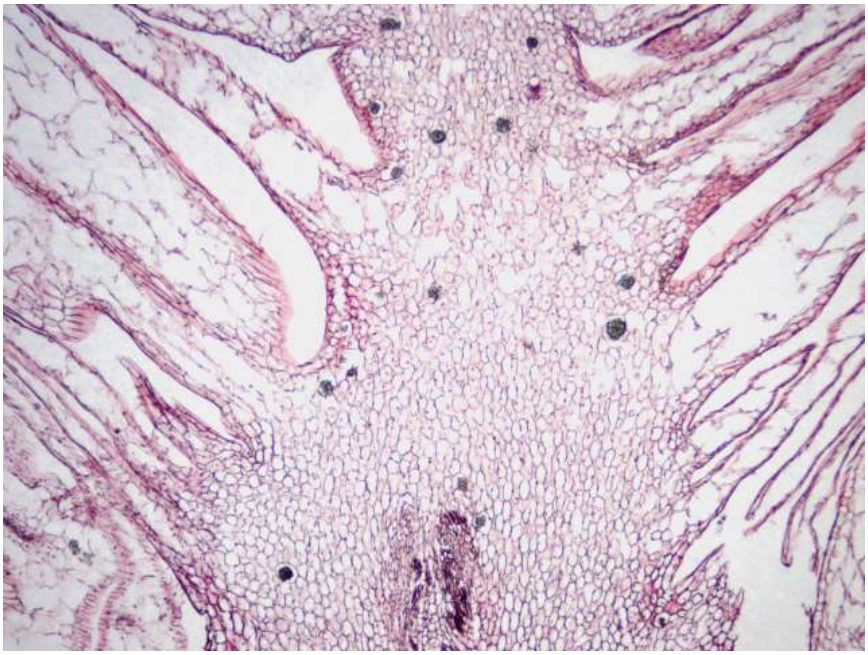

Figure 2. *In vivo* developed bud cluster connected to the spadix stem on ’Strauss’ cultivar

The leaf primordia and stem axis of the formula already contain differentiated tissues, vascular elements can be observed in the stem part (Figure 3).

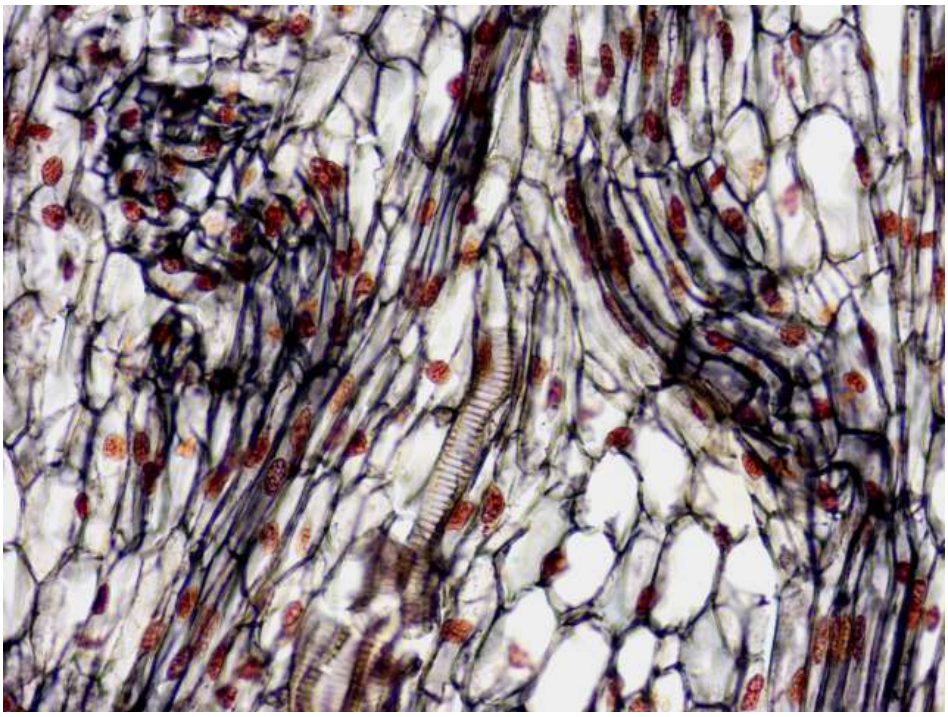

Figure 3. Vascular tissues in the stem part of the bud cluster

The leaf primordia have a thickened-walled single-row epidermis, stomatal pores are also found on the epidermis, and excretory idioblasts can be observed sporadically in the mesophyllum of the leaf primordia, with calcium oxalate raphide crystals characteristic to aroid species (Figure 4.)

Figure 4. Leaf primordia of the bud cluster

There is an apical meristem at the apex of the stem axis, and axillary bud meristems develop at the base of the leaf primordia at the top of the stem (Figure 5.).

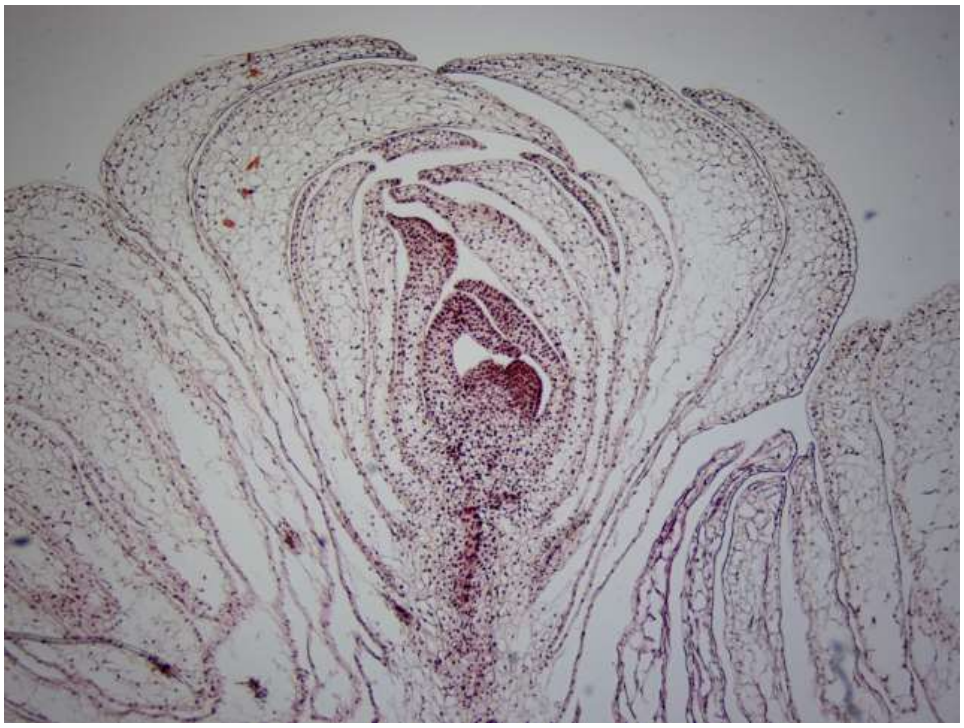

Figure 5. Apical meristem in the bud cluster

The anatomy of the bud clusters formed *in vitro* and then detached from the inflorescence and propagated further on *in vitro* media is the same.

Because the number of bud clusters formed *in vivo* was quite small, I did not have the opportunity to study the developmental process of bud cluster on the spadix. On the other hand, I followed the process of spadix development on young, developing inflorescences that did not show any sign of bud cluster from the outside, but I assumed that their presence may be histologically detectable at an early stage. I examined 'Petite' inflorescences aged 1–14 days from their appearance on the mother plant histologically, but found no signs of bud clusters developing in them.

Propagation possibilities of the bud clusters. For the propagation of bud clusters of the 'Petite' cultivar, 2 major series of experiments were performed on liquid medium, in the first case I studied the effect of 4 concentration levels of 4 types of cytokinins (benzyladenine - BA, benzyladenine riboside - BR, metatopolin - MT, thidiazuron - TDZ) on the culture rotator together with naphthaleneacetic acid, during all 16 treatments, evaluation was performed after 2 months when morphological parameters of the bud clusters were measured and their status was determined by classification on a scale of 1–5. In the second experiment, I investigated the multiplication in different liquid culture systems: working on a tissue culture rotor, orbital shaker, and a tube roller using the previous 4 concentration levels of TDZ. There were a total of 12 treatments here, and evaluation was also performed after 2 months.

The results of the first experiment investigating the effect of different cytokines are summarized in Table 1. The increase in the number of buds within the clusters is the primary indicator of multiplication, it is the largest under the influence of the two higher concentrations of BA and at every level of TDZ, in the case of TDZ no significant difference can be detected between the concentrations. Analyzing the peroxidase activity measured from the buds of state category 3 (healthy, actively multiplicating buds), it can be concluded that there is a significant difference between the groups according to the cytokinin types. The lowest POD activity was measured in the BA and TDZ groups, where the rate of proliferation was the highest, and a significantly higher stress

effect was measured in the BR and especially in the MT group. Examining the distribution of bud cluster status, there are treatments in the BA and TDZ groups that result in zero or minimal bud death. There is a significant difference between the mean status distributions of the treatments grouped according to cytokinines: BA causes minimal bud death in the stock, there is a high proportion of category 3 or 4 clusters (multiplicating buds or clusters showing signs of shoot regeneration) (Figure 6.), while BR shifts the distribution to the other direction: the proportion of buds that died or began to perish is larger. The MT and TDZ groups are in a transient state between the two endpoints, with no significant differences from either. 0.2 BA or 0.1 TDZ is recommended for propagation.

Table 1. Effect of different cytokinins in liquid media propagation of the bud clusters

cytokinin	bud number in the cluster			peroxydase activity		state categorisation (%) letters mean differences in the distribution of category data						
						1	2	3	4	5		
0.05 BA	4,6	cde	ab	12,9	a	0,0	0,0	96,4	3,6	0,0	ab	a
0.1 BA	6,3	abcd				0,0	0,0	82,6	17,4	0,0	ab	
0.2 BA	9,3	a				0,0	5,7	68,6	17,1	8,6	a	
0.5 BA	8,8	ab				0,0	58,8	41,2	0,0	0,0	d	
0.05 BR	3,7	ef	b	30,4	b	0,0	13,3	83,3	3,3	0,0	abc	b
0.1 BR	4,4	def				3,6	0,0	89,3	7,1	0,0	ab	
0.2 BR	3,7	ef				0,0	12,2	80,5	4,9	2,4	abcd	
0.5 BR	3,5	cdef				17,6	23,5	58,8	0,0	0,0	cd	
0.05 MT	4,5	cdef	ab	63,2	c	17,9	0,0	82,1	0,0	0,0	bcd	ab
0.1 MT	4,4	cdef				0,0	6,7	93,3	0,0	0,0	abc	
0.2 MT	5,8	bcd				14,3	17,1	57,1	11,4	0,0	abcd	
0.5 MT	3,1	f				0,0	6,3	87,5	6,3	0,0	abc	
0.05 TDZ	6,2	abcdef	a	14,6	a	0,0	23,1	53,8	15,4	7,7	abc	ab
0.1 TDZ	7,4	abcdef				0,0	0,0	92,6	7,4	0,0	ab	
0.2 TDZ	5,1	bcdef				7,7	34,6	53,8	3,8	0,0	cd	
0.5 TDZ	7,2	abc				0,0	6,7	80,0	10,0	3,3	ab	

In the second propagation experiment (Table 2.), where I investigated the effect of different devices during liquid culturing, the number of buds indicating proliferation was the same regarding the group averages of all TDZ concentrations for the rotator and the orbital shaker, while it was significantly lower for the tube roller. Of the individual concentrations, 0.1 TDZ provided the highest bud number, but this was significant only in the case of the orbital shaker, in the case of the rotator it did not differ statistically from the effect of the other concentrations. In the case of the tube roller, at the two higher examined TDZ concentrations all clusters were blackened / destroyed by the time of the evaluation, so no morphological measurements were performed there. The two lower TDZ concentrations also resulted in poorer results on the tube roller, not only the number of buds decreased, but also the diameter and weight of the clusters, the specific weight of the buds in the clusters and the peroxidase activity was significantly higher, which means higher stress in plant parts compared to the other method. There is no detectable difference between the state category distributions of the clusters due to the rotator and orbital shaker treatments, but the state category distribution of the clusters maintained with the tube roller shifts significantly, the buds die to a large extent. The differences between the concentrations are also clear: within each culturing method, increasing the concentration of TDZ results in a deterioration of bud states, an increasing number of clusters showing signs of blackening. Based on the conclusions drawn from the liquid culture experiments, thidiazuron at a concentration of 0.1 in combination with 0.1 NAA can be optimally used to grow bud clusters using a tissue culture rotator or an orbital shaker.

Table 2. Effect of different liquid culturing systems on the propagation of the bud clusters

	cytokinin	bud number in the cluster			peroxydase activity		state categorisation (%) letters mean differences in the distribution of category data						
							1	2	3	4	5		
RO	0.05 TDZ	7,2	ab	a	14,6	a	0,0	0,0	100,0	0,0	0,0	ab	a
RO	0.1 TDZ	9,4	ab				0,0	2,2	93,3	4,4	0,0	a	
RO	0.2 TDZ	6,0	bc				6,7	17,8	73,3	2,2	0,0	abcd	
RO	0.5 TDZ	5,2	bcef				4,4	37,8	55,6	2,2	0,0	cd	
OS	0.05 TDZ	4,3	cdef	a	17,7	ab	2,2	13,3	53,3	31,1	0,0	a	a
OS	0.1 TDZ	9,4	a				0,0	0,0	100,0	0,0	0,0	ab	
OS	0.2 TDZ	5,4	bce				0,0	11,1	88,9	0,0	0,0	abc	
OS	0.5 TDZ	5,5	bcd				4,4	33,3	55,6	4,4	2,2	bcd	
TR	0.05 TDZ	3,4	df	b	27,7	b	0,0	44,9	53,1	2,0	0,0	cd	b
TR	0.1 TDZ	3,1	d				2,1	52,1	45,8	0,0	0,0	d	
TR	0.2 TDZ	n/a			n/a		100	0	0	0	0		
TR	0.5 TDZ						100	0	0	0	0		

Figure 6. Bud clusters propagated on liquid culture media with BA

Shoot regeneration from the bud clusters. Shoot regeneration sometimes starts spontaneously from the bud clusters. According to my observations on the onset of shoot regeneration, the frequent division of clusters is not conducive to the process, and the duration of regeneration is prolonged in some cases (1-3 months). I have divided the shoot regeneration process into 6 phases (Figure 6.) Root formation almost always occurs in phase 6, but I have previously observed it in some cases starting from phase 2.

Figure 6. Shoot regeneration process from bud clusters

Because gibberellic acid was more likely to inhibit shoot conversion than auxin according to my previous experiences therefore, I tested several triazole-type (paclobutrazole, uniconazole, tebuconazole) and one pyrimidine carbinol (flurprimidol) compounds that cause, among other things, inhibition of gibberellin biosynthesis in plants, to examine whether they promote shoot regeneration on solid media. The inhibitors were tested in a concentration range, combined with cytokinins altogether in 30 different treatments (Table 3). Based on the results, some of these gibberellin biosynthesis inhibitors caused shoot regeneration already after 3 months. In the absence of cytokinin and auxin, paclobutrazol (PBZ) induced more significant regeneration at low concentration (0.25) (phases 4 and 5 combined: 28.2%) as early as 3 months, but as the concentration increased, an increasing proportion of bud clusters died (Table 2). Addition of 0.5 BA to PBZ treatments decreases the mortality rate with increasing PBZ concentration, and with 1 BA addition, this effect disappears, and shoot regeneration is highest in both cases at 0.25 PBZ. In the study of uniconazole, lower concentrations initiated shoot regeneration (phase 4 clusters), at the lowest concentration of 0.03125 the proportion of phase 4 clusters was 25%, but after 3 months there was no fully regenerated shoot, however, with the increase of concentration mortality of bud clusters also increased here. There was no significant difference between tebuconazole treatments - there was a good rate of regeneration at all concentrations (16.7-27.8%) and minimal mortality. Flurprimidol was tested at lower cytokinin levels than the other treatments, and the results showed that the mortality rate was also high here just like in the PBZ treatments at low cytokinin levels. Regarding the treatment groups, the best shoot regeneration with the lowest mortality out of the 4 gibberellin biosynthesis inhibitors was reached with the treatments 1 BA + tebuconazole, or 0.5 and 1 BA + paclobutrazole should be highlighted where a concentration of paclobutrazole is optimal at 0.25. It can be established that triazoles result in faster and greater shoot regeneration from buds, however, no growth regulator combination has been found so far that would result in an average of more than 25% more efficient shoot development in a short period of time (maximum 3 months). To investigate the induction of shoot regeneration, I also performed experiments where I examined the effect of changes in environmental parameters: I placed the cultures under green, red, blue wavelength light or in the dark, but these did not lead to results, either high or low temperature treatments.

Table 3. Examination of shoot regeneration with GA inhibitors

treatment	cytokinin	auxin	GA inhibitor	state categorisation (%) letters mean differences in the distribution of category data						
				1	2	3	4	5		
1	-	-	0.25 PBZ	6,5	2,2	63,0	13,0	15,2	a	d
2	-	-	0.5 PBZ	20,0	35,6	35,6	4,4	4,4	b	
3	-	-	1 PBZ	35,4	52,1	4,2	8,3	0,0	b	
4	-	-	2 PBZ	97,9	2,1	0,0	0,0	0,0	c	
5	0.5 BA	0.1 NES	0.25 PBZ	0,0	0,0	59,1	22,7	18,2	a	ab
6	0.5 BA	0.1 NES	0.5 PBZ	4,5	0,0	75,0	18,2	2,3	ab	
7	0.5 BA	0.1 NES	1 PBZ	8,9	16,1	50,0	23,2	1,8	b	
8	0.5 BA	0.1 NES	2 PBZ	16,3	44,2	32,6	7,0	0,0	c	
9	1 BA	0.1 NES	0,03125 PBZ	5,6	0,0	86,1	8,3	0,0	ab	ab
10	1 BA	0.1 NES	0,0625 PBZ	0,0	0,0	90,9	9,1	0,0	ab	
11	1 BA	0.1 NES	0,125 PBZ	0,0	16,7	63,9	19,4	0,0	ab	
12	1 BA	0.1 NES	0.25 PBZ	1,2	0,0	75,9	12,0	10,8	a	
13	1 BA	0.1 NES	0.5 PBZ	0,0	14,9	73,1	10,4	1,5	b	
14	1 BA	0.1 NES	1 PBZ	0,0	20,6	77,9	0,0	1,5	b	
15	1 BA	0.1 NES	2 PBZ	0,0	23,5	73,5	2,9	0,0	b	
16	1 BA	0.1 NES	0.03125 UNZ	0,0	0,0	75,0	25,0	0,0	a	b
17	1 BA	0.1 NES	0.0625 UNZ	0,0	0,0	91,7	8,3	0,0	a	
18	1 BA	0.1 NES	0.125 UNZ	0,0	0,0	86,1	13,9	0,0	a	
19	1 BA	0.1 NES	0.25 UNZ	6,9	1,4	86,1	4,2	1,4	ab	
20	1 BA	0.1 NES	0.5 UNZ	5,6	8,3	77,8	8,3	0,0	ab	
21	1 BA	0.1 NES	1 UNZ	15,2	18,2	63,6	3,0	0,0	bc	
22	1 BA	0.1 NES	2 UNZ	33,3	18,5	48,1	0,0	0,0	c	
23	1 BA	0.1 NES	0.03125 TBZ	0,0	5,6	69,4	13,9	11,1	a	a
24	1 BA	0.1 NES	0.0625 TBZ	2,8	8,3	72,2	8,3	8,3	a	
25	1 BA	0.1 NES	0.125 TBZ	0,0	5,6	66,7	16,7	11,1	a	
26	1 BA	0.1 NES	0.25 TBZ	0,0	2,8	77,8	8,3	11,1	a	
27	0.2 BA	0.1 NES	0.05 FPR	22,2	11,1	48,1	18,5	0,0	ab	c
28	0.2 BA	0.1 NES	0.1 FPR	6,7	16,7	50,0	26,7	0,0	a	
29	0.2 BA	0.1 NES	0.2 FPR	13,3	20,0	50,0	16,7	0,0	a	
30	0.2 BA	0.1 NES	0.5 FPR	22,2	55,6	18,5	3,7	0,0	b	

Enzymological measurements, physiological gibberellin activity assay. The peroxidase levels of *in vitro* bud clusters showing no signs of shoot regeneration were always higher than those measured in leaf *in vivo* or in the leaves of *in vitro* shoot cultures, they are statistically equal to those measured in the spadix before or after flowering (Figure 7.). On the other hand, the peroxidase level of buds starting shoot regeneration decreases to the same range which can be measured in leaves *in vivo*, or *in vitro* shoots and as the process of shoot regeneration progresses, it has a characteristic course: it first slightly rises and then decreases again after its completion. The high peroxidase activity of bud clusters may be explained by the formation of these formulas on the spadix, so the oxidative environment in them may be similar. During the generative phase of the plants the proportions and levels of growth regulators are altered which plays a role in establishing this higher oxidative environment. An increase in physiologically active gibberellin level is reported to be associated with an increase in the oxidative environment, and gibberellins also play a role in the establishment and maintenance of the generative state in plants belonging to the Araceae family. The physiological gibberellin activity measured in the leaves of non-flowering and flowering plants not treated with gibberellin shows a 36% increase in the 'Petite' variety and a 92% increase in the 'Bellini' variety based on the lettuce hypocotyl bioassay performed. During the shoot regeneration process of bud

clusters, the change in gibberellin activity is analogous to peroxidase activity according to my measurements. The bioassay method is not suitable for direct comparison of gibberellin activity in different organs (leaves, spadixes and bud formulas) because other components in extracts of different organs may also affect the growth of lettuce hypocotyl making the comparation impossible. I did not have the opportunity for instrumental analysis.

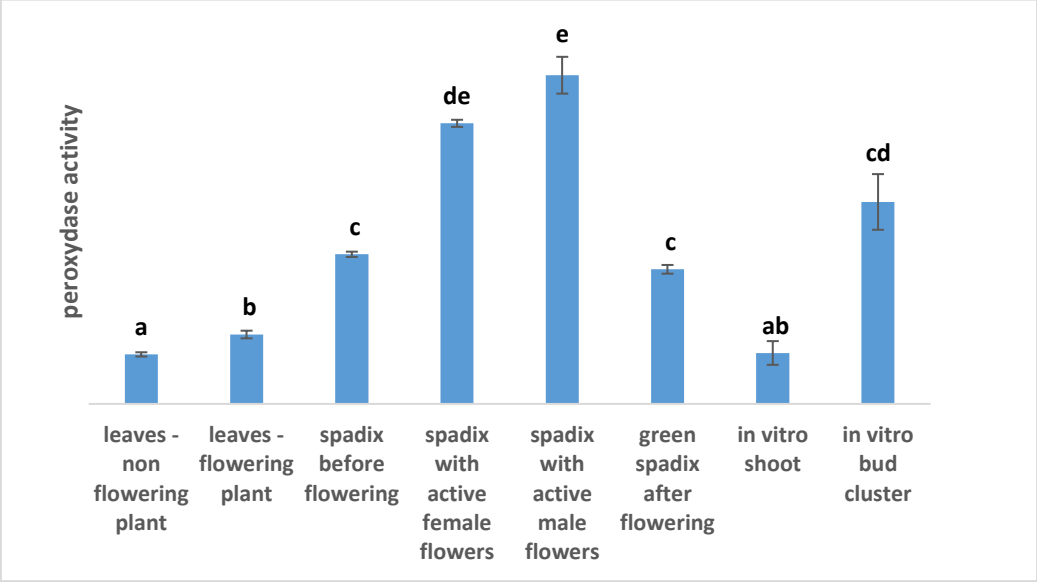

Figure 7. Peroxidase activity in different organs of Spathiphyllum ’Petite’ cultivar

Ploidy level examination. Since the bud clusters were always formed on inflorescences, the question arose that shoots derived from the bud clusters might be haploid. To clarify this issue, I collected root tip samples from *Spathiphyllum* 'Petite' and 'Cupido Compacto' parent plants and also from plants derived from bud clusters by both cultivar for measuring the size of the cell nuclei and counting the chromosome number and replicated the leaf epidermis (Figure 8.) to measure the size and density of stomatal pores in order to check the ploidy level. Chromosome counting has not been performed successfully, but cell nucleus size was comparable and no differences were detected. The size of the stomatal cells was accurately measured on the epidermis replicas made with preparative varnish. After statistical evaluation, I found that the stomatal sizes of bud cluster-derived 'Petite' and 'Cupido Compacto' plants do not differ significantly from the parent plants, and since the root-tip cell nucleus size are also similar, these bud cluster-derived plants are non-haploid. I repeated the examination a year later on freshly acclimatized plants of ‘Cupido Compacto’ cultivar, the results were showing the same again - there was no statistically difference in the size of the stomata.

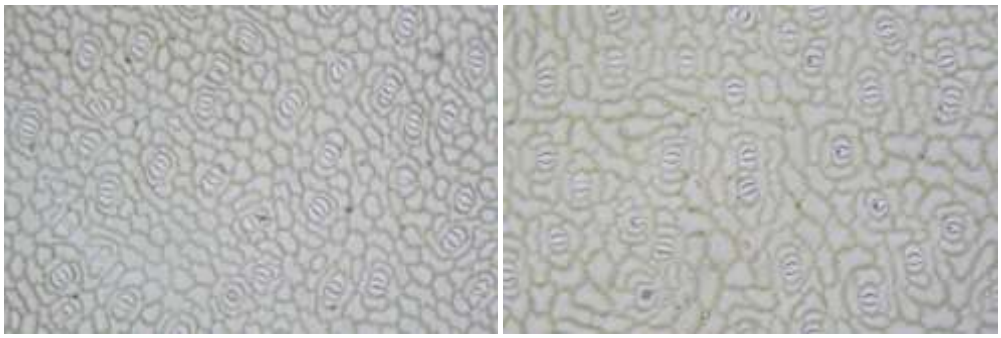

Figure 8. Stomata on the epidermis replica of ’Cupido Compacto’ mother plant (left) and of the bud cluster-derived regenerant (right)

Acclimation study. Bud cluster-derived 'Cupido Compacto' plants were acclimated (Figure 9.) and during acclimatization, I investigated the effect of a titanium compound, titanium ascorbate, on whether it increases the efficiency of acclimatization and the subsequent development of plants. Acclimatization was also successful with 100% result among the plants of the control group, the plants treated with titanium ascorbate showed neither macromorphological nor histological differences, but the test compound had a positive effect on their metabolism: the chlorophyll content of the treated plants increased. In addition, depending on the treatment concentration of titanium ascorbate, the proline content in the plants increased two and three times higher, respectively, and the size of stomatal openings decreased significantly, but the transpiration of the plants remained the same. Peroxydase activity in the treated plants increased significantly which together with the increased chlorophyll content shows the sign of increased stress tolerance. Treated and non-treated plants started to flower at the same time 6 months after being placed in the greenhouse. Titan-ascorbate treatment did not influenced the time of flowering.

Figure 9. Bud cluster-derived 'Cupido Compacto' plants before and 9 months after acclimatization

Management and maintenance of mother plant stocks. I only treated the purchased mother plants with gibberellin in the first year of the project to induce flowering, in the following years I allowed them to flower according to their natural cycle, that exogenous growth regulator treatment should not be an extra factor in the formation of bud formulas. A more serious problem arose once during the maintenance of the plant stock: the population of *Dieffenbachia* 'Green Magic' and 'Summer Style' was completely destroyed, half of the population of 'Bertina' was destroyed by systemic *Xanthomonas sp.* infection, I was unable to save the plants by spraying even streptomycin on them. The disease also appeared on *Anthurium* cultivars and on acclimatized young *Spathiphyllum* plants, but regular copper oxychloride spraying was used to limit its spread. The lost *Dieffenbachia* plants were not replaced, since bud formula development could not be induced in these taxa.

Limiting factors during the project. The research started with a 1-year delay at autumn of 2016, because tender projects were suspended in my institution due to institutional reorganization (joining another university), so I did not have access to the research grant until the handover processes took place.

Bud formulations appeared on the mother plants in a small percentage, and the size of the greenhouse area available to me did not allow for further expansion of the mother plant stock, so a small amount of sample was available for invasive studies. Most of the developed bud formulas were involved in *in vitro* propagation and shoot regeneration studies. In the case of Aracean plants, introduction into a sterile culture is a difficult task due to the frequent presence of endogenous bacteria, in several cases, this made it difficult to maintain the explants on the medium, and too strong disinfection leads to their destruction, so culture initiation from an already limited number of

inflorescence explants have been problematic on several occasions. The third limiting factor is the slow, multi-month process of shoot development from bud formulas, which has been accelerated by gibberellin biosynthesis inhibitors but would need further optimization. Due to the above mentioned reasons, the work was slower than planned, so I asked for an extension of the project.

Application of bud clusters in the micropropagation of *Spathiphyllum*. Summarizing the results achieved in the project, not all the details of the formation and development of the bud formula were fully clarified, as expected, but I provided valuable data on the usability of the formula. Micropropagation processes on solid media with shoot culture require quite a lot of manual labor, so they are expensive, but liquid culture can reduce costs, - in addition to having a positive effect on plant development-, because it may make the process partially or completely automated. According to my results, the bud clusters found in *Spathiphyllum* cultivars are excellent subjects for liquid culturing, they do not require any technical addition during liquid culture because they are able to float on the surface of the liquid medium. The propagation method based on bud clusters is using an organogenetic developmental pathway, thus minimizing the risk of somaclonal variability. The bud clusters can be used in a two-step propagation process where the propagation phase takes place in a liquid medium and the shoot regeneration is carried out on a solid medium similar to the methods used for orchid propagation with protocorm or protocorm like bodies. Due to their size and shape, they may also be suitable for use as an artificial-somatic seed after encapsulation, in which case they may be used immediately after automatized liquid phase propagation without the need of acclimation when encapsulated with a suitable growth regulator combination for shoot regeneration.

Publications

The multiplication possibilities of Spathiphyllum 'Petite' GGb clusters in liquid media was published as a conference abstract on the 13th International Scientific Conference – Biotechnology and quality of raw materials and foodstuffs (2018.09.17-19., Smolenice, Slovakia) conference.

The structure and development of bud clusters in *Spathiphyllum* and their usage in in vitro propagation is presented as a research paper, sent for publication to *Acta Scientiarum Polonorum Hortorum Cultus* (2019 IF: 0.616).

Results of the acclimatization of *Spathiphyllum* 'Cupido Compacto' with the help of titanium-ascorbate is presented also as a research paper, sent for publication to *Kertgazdaság*.

István Dániel Mosonyi

Budapest, 14th January 2021.