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Article

Response to *In Vitro* Micropropagation of Plants with Different Degrees of Variegation of the Commercial *Gymnocalycium* cv. Fancy (Cactaceae)

Carles Cortés-Olmos [1], Vladimir Marín Guerra-Sandoval [1], Carla Guijarro-Real [2], Benito Pineda [3,*], Ana Fita [1] and Adrián Rodríguez-Burruezo [1]

[1] Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Universitat Politècnica de València (UPV), Camino de Vera s/n, 46022 Valencia, Spain
[2] Department Biotechnology and Plant Biology, ETSIAAB, Universidad Politécnica de Madrid, Av. Puerta de Hierro, 2, Moncloa-Aravaca, 28040 Madrid, Spain
[3] IBMCP, Universitat Politècnica de València (UPV), Camino de Vera s/n, 46022 Valencia, Spain
* Correspondence: bpineda@btc.upv.es

Abstract: *Gymnocalycium* cv. Fancy is an ornamental cactus with a heterogeneous genetic background, which includes variegated variants whose in vitro culture response has not been studied. So plants exhibiting different degrees of variegation (from 0% to 100%) were also classified by their initial size and used to obtain different types of explants (apices, central discs, epicotyls and hypocotyls). The effects of three plant growth regulators (BAP at 8µM, KIN at 4µM and TDZ at 1µM) was evaluated. The response of explants was measured in the number of shoots, calluses and rhizogenesis events per explant; as well as the appearance of variegated shoots with specific color percentages. Central discs treated with 1 µM TDZ provided the best results in terms of shoot production. Additionally, a correlation was observed between the type of activated areole (green, mixed or fully colored) and the percentage of color of the obtained shoots, enabling precise explant selection based on desired morphological characteristics. Additionally, the appearance of shoots with different colors confirms the possibility of selecting new lines from this cultivar. These findings could be of great value not only for breeding and multiplication of ornamental cactaceae but also other edible species and relatives.

Keywords: citokinins; colored cactus; organogenesis; plant growth regulators; cactus areolas; explant

1. Introduction

Variegated plants represent a significant part of the ornamental plant market due to their aesthetic appearance [1]. This variegation results from a partial or total deficit of chlorophyll in certain regions of the plant, leading to colorations ranging from yellowish to whitish. The presence of leaves and/or stems with a different color or coloring patterns than those typical of the original species gives variegated plants a greater visual beauty and ornamental value, making them highly valued among both, amateur and professional gardeners or floriculturists [2,3]. Therefore, although these plants tend to be less vigorous than the non-variegated ones, due to their lower amount of chlorophyll, which affects their photosynthetic capacity [4], it is not surprising that commercial nurseries work intensively to obtain plants with new colorations or patterns to annually introduce into the market.

Nowadays, the presence of a wide range of variegated plants, including popular ones like pothos, alocasias and monsteras, reflects their significance in gardening and landscaping. Also, certain cactus cultivars developed from *Gymnocalycium mihanovichii* (Frič and Gürke) Britton and Rose, are commercially important due to their diverse coloration [5] (**Figure 1**). Many of these variants

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are clonally propagated through grafting [6,7], although the success of this process can be influenced by the ability of the parent plants to produce shoots with the expected patterns of coloration or color percentage.

Figure 1. Commercial selections of *Gymnocalycium mihanovichii* with different colorations and color distribution patterns.

The grafting technique is widely used in the mass propagation of cacti due to its advantages against plants grown from their roots [8]. Grafting allows: (i) enhanced plant development, (ii) increased shoot production, (iii) accelerate blooming, reducing the time between generations and (iv) intensify flowering (i.e. more flowers per season) [9]. Additionally, rootstocks are usually more vigorous and resistant to humidity, pests and diseases, minimizing losses from rot and simplifying their cultivation [8,9].

Nevertheless, grafting may alters the natural morphology of the plants, leading to an atypical appearance that may not appeal to collectors, who prefer visually natural plants capable of growing on their own roots. Completely achlorophyllous plants must remain grafted due to their inability to photosynthesize [4,10], so they are generally unpopular among collectors. This situation underscores

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the need for protocols to optimize the production processes of cacti with specific percentage of colorations that are able to develop on their own roots. Such protocols could significantly benefit large-scale cactus producers by improving efficiency and allowing for the selection of plants with desired variegation levels for the collector market.

In this context, *in vitro* cultivation can play a determinant role, since the regenerative capacity of different structures (mainly areoles) has been observed in a range of cacti species [7,11–13]. However, most of these studies have been focused on edible species that have interest on food industry, such as pitahaya and prickly pear [13–20]. Therefore, there is a lack of information regarding many ornamental species that could have a significant impact on the market, in particular when propagating variegated individuals or those with particular color patterns, as in most cases the cellular mechanisms that cause them are unknown [21].

Thus, the study focuses on the *in vitro* response of *Gymnocalycium* plants with varying degrees of variegation (**Figure 2**). Therefore, the objective of the experiment is to establish an efficient protocol for *in vitro* propagation of *Gymnocalycium* cv. Fancy plants with varying degrees of variegation. This will be achieved through evaluating the organogenic response and *in vitro* behavior of diverse explants (apical, central disc, hypocotyl, and epicotyl) from plants of different sizes (small, medium, and large) under three previously tested plant growth regulators (PGRs) used on chlorophyll-containing plants of the same cultivar [22].

Figure 2. Morphological heterogeneity observed in one-year-old *Gymnocalycium* cv. Fancy shoots obtained *in vitro* and subsequently acclimatized. **(a, b)** Expected morphologies within the usual range of variation of the cultivar. **(c, d)** Unexpected morphologies obtained, monstrous **(c)** and caespitose forms **(d)**. **(e)** Variegated plants with different degrees of variegation.

The aim is to determine the effect of these plant growth regulators on variegated plants and the relationship between the initial plants' variegation proportion and the productivity of shoots with varying degrees of variegation. This information could be highly relevant in a commercial context, whether it improves graft propagation efficiency or optimizes the production of partially variegated plants for cactus collectors. Furthermore, the results could be applicable to other cactus species that may attract interest from consumers and collectors.

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2. Results and Discussion

2.1. Explant Activation

The number of explants that resulted in some type of response (whether it be the formation of shoots, callus, or both), as well as their proportion relative to the total number of explants included for each factor and evaluated variable, are shown in **Table 1**. It is observed that the presence of TDZ1 in the medium significantly favored the activation of explants, with a response rate of 83.11%, while the other treatments (BAP8, 60.53%; KIN4, 50.67%) showed a lower activation efficiency, but slightly higher than those observed in the control group (46.77%) (**Table 1**). The high activation capacity of TDZ compared to other growth regulators, both in generating callus or forming shoots, has been reported in various studies with other cactus species [6,8], although it was particularly prominent in previous trials using similar *Gymnocalycium* cv. Fancy plants [22]. Thus, the repeatability of the response is confirmed in this trial.

Table 1. Percentage of activated explants as a function of evaluated factors at the 5th month.

Factor	Total Number of Explants	Activated Explants	% of Response [1]
Treatment [2]			
BAP8	152	92	60.53 a
KIN4	150	76	50.67 a
TDZ1	148	123	83.11 b
CONTROL	124	58	46.77 a
Plant Size			
Large	96	67	69.79 b
Medium	332	228	68.67 b
Small	146	54	36.99 a
% of Color			
0	220	136	61.82 b
25	110	74	67.27 b
50	60	41	68.33 b
75	156	95	60.90 b
100	28	3	10.71 a
Type of Explant			
Apical	214	137	64.02 b
Central Disc	214	158	73.83 c
Epicotyl	73	27	36.99 a
Hypocotyl	73	27	36.99 a
Total	**574**	**349**	**60.8**

[1] Values followed by the same letter are not statistically different for $p = 0.05$ according to the Student–Newman–Keuls; [2] BAP8 = 6-Benzylaminopurine at 8 µM; KIN4 = Kinetin at 4 µM; TDZ1 = Thidiazuron at 1 µM; CONTROL = Control explants grown in absence of PGRs.

On the other hand, there was no difference in the activation capacity of explants obtained from medium-sized plants (69%) or large plants (70%), although the explants from hypocotyl and epicotyl derived of smaller plants showed a lower response (37%) (**Table 1**). Furthermore, apical and central disc explants, which were obtained from longer plants, also showed high rates of activation (64-74%)(**Table 1**). These findings suggest that hypocotyl and epicotyl explants, composed of younger and less mature tissues, are not so efficient in activing their areoles with the only use of cytokinins,

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although specific combinations of auxins and cytokinins may increase efficiency, as it has been observed in other species [23–25]. In fact, some authors reported responses using different auxin/cytokinin ratios [26–31]

Considering the variegation percentage of the starting plants, it was found that the absence of chlorophyll was extremly limiting. Thus, fully variegated explants responded considerably worse (11% approximately) to treatments compared to plants with chlorophyll tissues (with a response rate > 60%), regardless of their color proportion (**Table 1**). This fact demonstrate that the total absence of chlorophyll hinders the normal development of plants on their own roots, due to the inability to carry out photosynthetic activity, which leads to their maintenance and propagation through grafting. This study has shown that fully variegated plants also exhibited very limited *in vitro* development, with many explants degenerating and others unable to grow beyond 8 mm. Probably, the inability to generate photo-assimilates might cause on these already delicate plants, a higher sensitivity to cuttings as it has been observed in *Agave angustifolia* Haw. albino variant somaclones [21].

2.1.1. Calli Production

The production of calli was found to be closely related to the presence of TDZ1 in the culture medium (**Table 2**), as have been reported in previous works [32–34]. From the beginning of the induction period, calli formation in explants grown under TDZ1 was higher than those observed in other PGRs and the control group (**Table 2**). Although activation in the presence of BAP8 was higher than that observed in the presence of KIN4 (with values very similar to the control group), the callus formation observed in these groups were minimal (**Table 2**). These results are in agreement with Giusti et al. [6], who reported a positive effect of TDZ on callus formation and shoot hyperhydration in *Escobaria minima* (Baird) D. Hunt, *Mammillaria pectinifera* and *Pelecyphora aselliformis*, while the presence of BAP and KIN favoured shoot formation.

Table 2. Average number of calli with their standard errors obtained monthly per factor and condition.

Factors	Cases	Induction Period in Presence of PGRs [1]				Development Period in Absence of PGRs			
		1st Month		2nd Month		3rd Month		4th Month	
		p-Value	Average [4]	*p*-Value	Average	*p*-Value	Average	*p*-Value	Average
Treatment [2]		0.000 *		0.000 *		0.000 *		0.000 *	
BAP8	92		0.28±0.10 b		0.17±0.06 a		0.03±0.02 a		0.00±0.00 a
KIN4	76		0.01±0.01 a		0.03±0.02 a		0.00±0.00 a		0.07±0.03 a
TDZ1	123		0.55±0.09 c		1.12±0.12 b		0.89±0.12 b		0.41±0.08 b
CONTROL	58		0.02±0.02 a		0.00±0.00 a		0.00±0.00 a		0.00±0.00 a
Plant Size [3]		0.949		0.041 *		0.504		0.37	
Large	67		0.36±0.13 a		0.48±0.11 ab		0.19±0.07 a		0.17±0.05 b
Medium	228		0.25±0.05 a		0.50±0.07 b		0.39±0.07 a		0.21±0.04 b
Small	54		0.26±0.08 a		0.17±0.07 a		0.20±0.09 a		0.07±0.07 a
% of Color		0.12		0.045 *		0.045 *		0.049 *	
0	136		0.23±0.06 a		0.34±0.08 a		0.33±0.08 ab		0.24±0.06 b
25	74		0.26±0.07 ab		0.69±0.12 b		0.57±0.15 b		0.22±0.08 ab
50	41		0.61±0.22 b		0.51±0.15 ab		0.07±0.04 a		0.02±0.02 a
75	95		0.21±0.06 a		0.39±0.09 a		0.22±0.06 a		0.05±0.04 a
100	3		0.33±0.33 ab		0.33±0.33 ab		0.33±0.33 ab		0.00±0.00 a
Type of Explant		0.000 *		0.000 *		0.001 *		0.043 *	
Apical	137		0.55±0.09 b		0.77±0.10 b		0.55±0.10 b		0.26±0.07 c
Central Disc	158		0.04±0.02 a		0.26±0.05 a		0.17±0.05 a		0.10±0.03 a
Epicotyl	27		0.48±0.15 b		0.19±0.08 a		0.37±0.17 ab		0.15±0.15 ab
Hypocotyl	27		0.04±0.04 a		0.15±0.12 a		0.04±0.04 a		0.00±0.00 a
Total	**349**		**0.28±0.04**		**0.45±0.05**		**0.32±0.05**		**0.16±0.03**

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[1] PGRs: Plant Growth Regulators. [2] Treatments: BAP8 (6-Benzylaminopurine at 8 µM), KIN4 (Kinetin at 4 µM) and TDZ1 (Thidiazuron at 1 µM). [3] Plant sizes: Large (12-16 mm); Medium (8-12 mm); Small (4-8 mm). [4] Values followed by different letters are statistically different for p =0.05 according to Student-Newman-Keuls. * Significant results at $p \leq 0.05$.

During the development period in absence of PGRs, the number of observed calluses tended to decrease in all treatments (except those exposed to KIN4), with a considerably reduction in the case of TDZ1 (**Table 2**). This effect would be related to the removal of hormones after the induction period, since callus formation is usually favored by the presence of cytokinins in the medium [8,32,35]. Through subsequent subcultures, the callus response would gradually decrease, stimulating the structuring and organization of calluses into defined shoots, as observed in other works [22,32–34]. From the fourth month of the trial on, no variations were detected in the number of calluses present on the explants.

The process of callogenesis was significantly higher in explants from medium and larger plants than in epicotyl and hypocotyl explants (**Table 2**). These differences were mainly due to the higher response of apical explants compared to epicotyl explants, although comparatively, central disc explants also showed better responses than hypocotyls (**Table 2**). Moreover, apices from medium-sized plants provided better results than those from large plants. This fact is usual with cacti, where it has been observed that younger areoles are more sensitive to hormonal treatments than older ones [36]. In fact, most micropropagation protocols in *Opuntia* and *Hylocereus* are based on the use of young plants or cladodes [14–16,37].

Regarding to the production of callus based on the variegation percentage of the initial plants, the explants from the control group and the group with 25% variegation showed a higher average number of calluses at the end of the trial compared to the explants with a higher percentage of coloration (**Table 2**). Although statistically significant differences were not observed during the induction period, the development of the explants in the following months varied depending on their color percentage. In fact, plants with lower variegation (control group and 25% variegation) reduced the number of detectable calluses to a lesser extent (45%) than the other groups with higher degrees of variegation (50%, 75%, and 100%), resulting in a 94% reduction of callus presence. These results suggest that plants with a higher percentage of chlorophyll-containing tissue are more stable, and after a callogenic process, the possibility of reverting calluses to defined shoots is lower. Therefore, calluses obtained from explants with a higher degree of variegation would exhibit greater reversibility to shoots, presumably due to being more sensitive to cytokinins than calluses from chlorophyll-containing tissue. This fact was also observed by Rouinsard *et al.* [38], who reported different responses and behaviours when compared *in vitro* micropropagation of *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and *Cordyline australis* 'Pink Passion', three cultivars with variegated foliage. In this case, they confirmed that the variegation stability was genotype-dependent and highly related to the ability of the explants to be propagated by adventitius meristems [38].

2.1.2. Shoot production

The average of the monthly shoot production per explant for each evaluated factor was recorded in the **Table 3**. The results demonstrate a significant contribution of hormonal effect on explant activation, both during the induction period and the development period in the absence of regulators. In the first two months of cultivation, under hormone conditions, explants treated with TDZ1 showed a lower shoot production compared to other treatments and the control group. However, after subculturing in a medium without regulators, TDZ1-activated explants started responding positively and eventually showed the best average results at the end of the assay **Table 3**. This increase in shoot numbers is closely linked to the observed reduction in the number of calli.

Therefore, the total shoot count at the end of the trial is not only due to new areola activation in explants but also to callus structuring and differentiation into shoots. Similar results were obtained in the evaluation of chlorophyllous plants [22] and *Rauvolfia serpentina* (L.) Benth. ex Kurz plants [32], where the effect of TDZ1 agrees with out findings. In contrast, BAP8 and the control group resulted

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in very similar shoot productions, while KIN4 showed slightly lower values (**Table 3**). Considering that responses to exogenous hormones could differ depending on the species and explant source [8,39,40], TDZ1 treatment would be the most efficient for propagating this type of plant material. Probably, the ability of TDZ to alterate endogenous cytokinin metabolism [41–45] could explain its greater activation ability in explants compared to BAP8 or KIN4.

Table 3. Average number of shoots (±SE) obtained monthly per factor and condition.

Factors	Cases	Induction Period in Presence of PGRs [1]				Development Period in Absence of PGRs [1]					
		1st Month		2nd Month		3rd Month		4th Month		5th Month	
		p -Value [4]	Average	*p*-Value	Average	*p*-Value	Average	*p*-Value	Average	*p*-Value	Average
Treatment [2]		0.015 *		0.000 *		0.142		0.433		0.000 *	
BAP8	92		0.13±0.04 b		0.77±0.13 b		1.55±0.15 ab		2.09±0.15 a		2.33±0.15 a
KIN4	76		0.12±0.06 b		1.00±0.12 b		1.51±0.12 ab		1.80±0.12 a		1.97±0.11 a
TDZ1	123		0.00±0.00 a		0.31±0.07 a		1.30±0.11 a		2.19±0.12 a		2.85±0.13 b
CONTROL	58		0.12±0.07 b		0.93±0.21 b		1.83±0.26 b		2.16±0.26 a		2.43±0.25 a
Explant Size [3]		0.589		0.693		0.529		0.167		0.199	
Large	67		0.13±0.06 a		0.79±0.07 a		1.69±0.21 a		2.21±0.21 a		2.58±0.20 a
Medium	228		0.07±0.02 a		0.68±0.07 a		1.47±0.09 a		2.11±0.09 a		2.49±0.10 a
Small	54		0.07±0.04 a		0.56±0.12 a		1.41±0.14 a		1.74±0.16 a		2.13±0.16 a
% of Color		0.196		0.149		0.042 *		0.622		0.792	
0	136		0.12±0.04 b		0.73±0.10 a		1.40±0.14 a		2.14±0.14 a		2.46±0.13 a
25	74		0.00±0.00 a		0.57±0.13 a		1.34±0.15 a		2.04±0.15 a		2.42±0.18 a
50	41		0.07±0.04 ab		0.98±0.26 a		2.05±0.25 b		2.22±0.23 a		2.66±0.25 a
75	95		0.10±0.05 ab		0.56±0.09 a		1.52±0.12 ab		1.92±0.14 a		2.36±0.12 a
100	3		0.00±0.00 ab		1.67±0.88 a		2.00±0.58 ab		2.67±0.88 a		3.00±1.00 a
Type of Explant		0.081		0.047 *		0.086		0.013 *		0.005 *	
Apical	137		0.13±0.04 b		0.91±0.13 b		1.77±0.15 b		2.39±0.14 b		2.80±0.15 b
Central Disc	158		0.04±0.02 a		0.53±0.07 a		1.30±0.10 a		1.91±0.10 a		2.26±0.10 a
Epicotyl	27		0.11±0.08 ab		0.70±0.18 ab		1.37±0.19 ab		1.70±0.24 a		2.30±0.27 ab
Hypocotyl	27		0.00±0.00 ab		0.41±0.15 a		1.44±0.22 ab		1.78±0.22 ab		1.96±0.19 a
TOTAL	**349**		**0.08±0.02**		**0.69±0.06**		**1.50±0.08**		**2.07±0.08**		**2.45±0.08**

[1] PGRs: Plant Growth Regulators. [2] Treatments: BAP8 (6-Benzylaminopurine at 8 µM), KIN4 (Kinetin at 4 µM) and TDZ1 (Thidiazuron at 1 µM). [3] Plant sizes: Large (12-16 mm); Medium (8-12 mm); Small (4-8 mm). [4] Values followed by different letters are statistically different for p =0.05 according to Student-Newman-Keuls. * Significant results at $p \leq 0.05$.

There were no statistically significant differences regarding the initial size of the plant material, although the average values obtained from small explants were slightly lower (**Table 3**). Therefore, even though the activation percentage of explants from small plants was noticeably lower compared to those from medium and large plants (**Table 1**), once activated they enabled a productivity similar to that observed in explants from larger plants (**Table 3**).

When comparing the different types of explants, the highest productivity was observed in apical explants (2.80) from the beginning of the trial. The productivity of epicotyls (2.30) and central discs (2.26) were very similar, while hypocotyls provided a slightly lower average (1.96) (**Table 3**). These results highlight the potential of epicotyl explants compared to apical and central disc explants, suggesting that they could be very interesting in commercial terms, with a more optimized protocol of activation. The ability to use small seedlings as source material for micropropagation could reduce considerably the time between *in vitro* cultivation cycles, making the process faster and more profitable.

Related to the influence of the color percentage of the starting material, no significant differences were observed between the various color groups and the control group (0% presence of color) (**Table 3**). This situation demonstrates that the presence of variegation in plants does not limit their areolar response capacity. Even fully variegated explants were able to activate an average of three areolas

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per explant by the end of the trial (**Table 3**). Therefore, the limitation lies in the activation capacity of the explants (**Table 1**), as once activated, they could potentially offer similar responses.

2.1.3. Efficiency Related to Areolar Activation

Since the number of areolas available on each type of explant is variable (**Table 4**), the theoretical response may also differ. In addition to evaluating productivity in number of calluses and number of shoots, the efficiency of the explants was also assessed on the percentage of activated areolas compared to the total number on each explant (**Table 5**). From this perspective, some of the results observed in the previous section were maintained, such as the higher efficiency of TDZ1 in areolar activation, the better response of medium and large-sized explants compared to small ones, and the activation ability regardless of the initial variegation degree (except for fully variegated plants) (**Table 5**). However, we found that the efficiency of central discs nearly doubled those of apical and hypocotyl explants, while epicotyls showed a significantly lower percentage of areolar activation compared to the rest (**Table 5**). Considering the contribution of the hormonal treatment and the type of explant, a graphical representation of productivity and efficiency results was conducted to show in a more detailed way the interaction of these main factors (**Figure 3**).

Table 4. Average number of areoles per explant according to initial plant size and explant type.

Factor	Cases	Average [1]
Plant Size		
Large	96	9.30 ± 0.43 a
Medium	332	9.37 ± 0.27 a
Small	146	10.21 ± 0.54 a
Type of Explant		
Apical	214	13.15 ± 0.25 b
Central Disc	214	5.51 ± 0.13 a
Epicotyl	73	15.30 ± 0.51 b
Hypocotyl	73	5.12 ± 0.43 a

(1) Values followed by the same letter are not statistically different for p = 0.05 according to the Student–Newman–Keuls.

Table 5. Average number of the percentage of activated areoles with their standard errors obtained monthly by factor and condition.

Factors	Cases	Induction Period in Presence of PGRs [1]		Development Period in Absence of PGRs					
		1st Month		2nd Month		3rd Month		4th Month		5th Month	
		p-	Average [4]	*p*-	Average	*p*-	Average	*p*-	Average	*p*-	Average
Treatment [2]		0.000		0.006		0.015		0.207		0.006	
BAP8	92		3.263±0.765		10.291±1.572 a		18.488±1.897 a		26.509±2.364 a		29.313±2.188 a
KIN4	76		0.961±0.475 a		14.414±1.968		22.767±2.491		29.755±3.065 a		32.667±3.151 a
TDZ1	123		4.629±0.718 c		16.293±1.559 b		27.384±1.854 b		33.533±2.474 a		43.107±3.222 b
CONTROL	58		1.629±0.931		11.336±2.336 a		22.995±3.162		26.996±3.164 a		30.727±3.040 a
Explant size		0.707		0.002		0.009		0.000		0.000	
Large	67		4.071±1.152 a		14.692±2.102 b		22.715±2.375 b		27.452±2.607 b		32.699±2.463 b
Medium	228		2.899±0.458 a		14.929±1.184 b		25.679±1.508 b		33.634±1.850 b		39.160±2.155 b
Small	54		1.914±0.491 a		5.845±1.129 a		14.006±1.424 a		16.344±1.651 a		21.191±2.186 a
% of Color		0.742		0.379		0.849		0.003		0.136	
0	136		3.298±0.704 a		13.574±1.599 a		24.742±2.113 a		35.214±2.553 b		39.862±2.770 b
25	74		2.250±0.579 a		15.962±2.002 a		23.722±2.116 a		28.844±2.056 b		33.596±2.282 b
50	41		4.058±1.209 a		14.706±2.380 a		22.836±2.850 a		24.828±2.959		28.937±2.981 b
75	95		2.604±0.677 a		10.880±1.503 a		21.378±1.946 a		25.246±2.675		32.749±3.437 b
100	3		2.778±2.778 a		13.333±7.265 a		15.185±5.614 a		16.852±5.830 a		19.630±8.021 a
Type of		0.000		0.003		0.001		0.000		0.000	
Apical	137		4.670±0.641 b		14.150±1.118 b		20.025±1.416 b		22.614±1.407 b		25.824±1.545 b

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Central Disc	158	1.861±0.587 a	15.504±1.664 b	29.325±2.001 c	40.568±2.448 c	47.984±2.760 c
Epicotyl	27	3.416±0.800 b	5.605±1.296 a	11.105±1.559 a	11.926±1.780 a	16.124±2.286 a
Hypocotyl	27	0.412±0.412 a	6.085±1.874 a	16.906±2.278	20.763±2.538	26.258±3.504
Total	349	2.972±0.380	13.478±0.905	23.304±1.127	29.772±1.371	35.140±1.560

(1) PGRs: Plant Growth Regulators. (2) Treatments: BAP8 (6-Benzylaminopurine at 8 μM), KIN4 (Kinetin at 4 μM) and TDZ1 (Thidiazuron at 1 μM). (3) Plant sizes: Large (12-16 mm); Medium (8-12 mm); Small (4-8 mm). (4) Values followed by different letters are statistically different for p =0.05 according to Student-Newman-Keuls.

The combination of both factors showed that, in absence of PGRs, explants generally exhibited a lower shoot production compared to that observed after hormonal treatments. However, in the case of apical explants, the control group showed the best results (**Figure 3a**). This fact in apical explants could be attributed to changes in the endogenous concentration of phytohormones that naturally activate this tissue after damage to the apical bud, which is common in cacti [33,34]. Therefore, this response would be related to an intrinsic ability to activate dormant buds in that specific tissue fraction and not to the presence of a specific hormone in the medium.

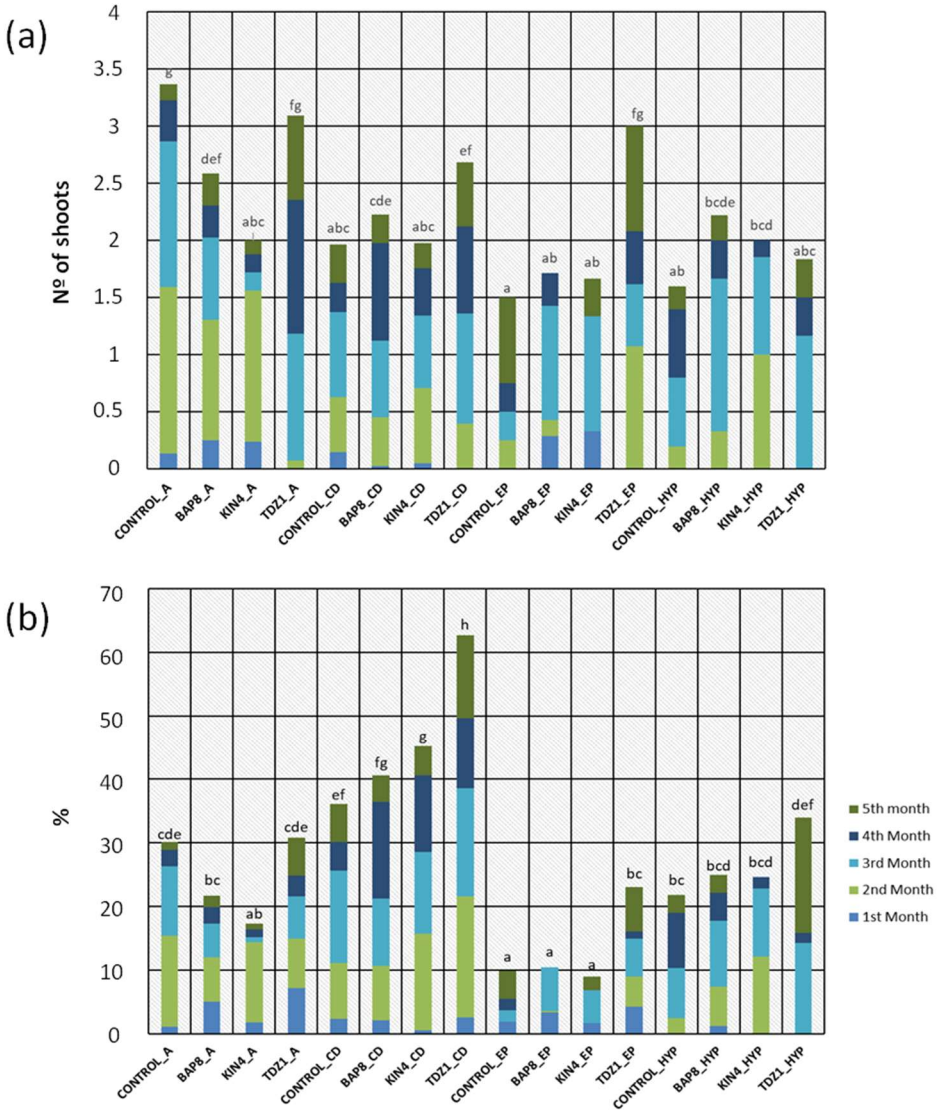

Figure 3. (a) Average number of shoots obtained for each "Hormone + Explant Type" combination during each month of cultivation. **(b)** Average percentage of areoles activated by each "Hormone + Explant Type" combination during each month of cultivation**.** Hormone: CONTROL (control group), BAP8 (6-Benzylaminopurine), KIN4 (Kinetin) and TDZ1 (Thidiazuron). Numbers following the conditions indicate the hormonal concentration (1, 4 or 8 μM). Capital letters indicate the explants used in each combination: A (apical),

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CD (central discs), EP (epicotyl) and HYP (hypocotyl). Letters (a, b, c, d, e, f) above the bars represent significant differences based on sample means at the fifth month of evaluation, for $p = 0.05$ according to the Student–Newman–Keuls test.

In the evaluation of the average percentage of activated areolas, TDZ showed its greater ability to induce response in all types of explants. Furthermore, a higher efficiency was observed in central disc explants compared to the rest of the explants (**Figure3b**), even in those from the control group. The similarity in results between central discs and apices from the control groups reinforces the hypothesis of natural activation of dormant buds in plants with damaged apical meristems [33,34]. However, a slightly more efficient response was evident in areolas from the central region of the plant (**Figure 3b**). These results are in agreement with previous trials conducted on chlorophyllous plants [22], although in this case, splitting the apices into two (thus eliminating apical dominance phenomena) allowed for a more comparable assessment of their efficiency compared to central discs.

Finally, the specific combination of TDZ1 and central discs resulted in the best trial results, with percentages nearly doubling those obtained using other types of explants (**Table 5** and **Figure 3b**). Considering the significant activation capacity of central disc explants (**Table 1**), their high potential in activating their areolas in the presence of TDZ1 (**Figure 3b**), and thus their productivity in a strict sense (**Figure 3a**), the efficiency of this protocol would stand out above any other possible combination.

2.2. Root Emergence

Considering that the presence of roots could influence their response in generating shoots or calluses [46–48], the root emergence was recorded during the first three months of *in vitro* cultivation, and some of the explants showed rhizogenesis. In general terms, the presence of TDZ1 in the medium had a highly detrimental effect on root formation (with root emergence being minimal), while the lack of cytokinins favored root emission. In fact, the control group showed the highest values in the trial, followed by treatments with KIN4 and BAP8 (**Table 6**). TDZ is a synthetic hormone with both auxinic and cytokinin activity [49] that appears to have the ability to block or inhibit natural rooting mechanisms, as observed in previous studies [22]. From this perspective, the artificial origin of hormones could influence root emergence in the explants, as similar results have been obtained in other works with 2,4D [50]. However, the use of natural hormones like KIN or BAP seems to have a less negative impact, although they still provide lower rooting values compared to those observed in the control explants.

Table 6. Average and standard error of root emission frequency (calculated as number of rooted explants based on the total number of explants) obtained monthly by factor and condition.

Factors	Cases	Induction Period in Presence of PGRs[1]				Development Period in Absence of	
		1st Month		2nd Month		3rd Month	
		p-Value	Average [5]	*p*-Value	Average	*p*-Value	Average
Treatment [2]		0.000		0.000		0	
BAP8	92		0.228±0.044 b		0.348±0.050 b		0.413±0.052 b
KIN4	76		0.368±0.056 c		0.461±0.058 bc		0.487±0.058 bc
TDZ1	123		0.008±0.008 a		0.008±0.008 a		0.024±0.014 a
CONTROL	58		0.517±0.066 d		0.569±0.066 c		0.552±0.066 c
Explant size [3]		0.1329		0.2903		0.5802	
Large	67		0.298±0.056 ab		0.343±0.058 a		0.343±0.058 a
Medium	228		0.232±0.028 b		0.294±0.030 a		0.294±0.030 a
Small	54		0.130±0.046 a		0.204±0.055 a		0.370±0.066 a
% of Color		0.0006		0.0008		0.0165	
0	136		0.346±0.041 b		0.412±0.042 b		0.404±0.042 c
25	74		0.189±0.046 a		0.257±0.051 a		0.270±0.052 a
50	41		0.195±0.063 a		0.244±0.068 a		0.366±0.076 b

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75	95	0.116±0.033 a	0.168±0.039 a	0.200±0.041 a
100	3	0.000±0.000 ab	0.000±0.000 ab	0.333±0.333 ab
TE[4]		0	0	0
Apical	137	0.431±0.042 b	0.511±0.043 b	0.504±0.043 b
Central Disc	158	0.089±0.023 a	0.127±0.027 a	0.133±0.027 a
Epicotyl	27	0.222±0.082 a	0.296±0.090 a	0.519±0.098 b
Hypocotyl	27	0.037±0.037 a	0.111±0.062 a	0.222±0.082 a
Total	349	0.229±0.022	0.289±0.024	0.315±0.025

[1] PGRs: Plant Growth Regulators. [2] Treatments: BAP8 (6-Benzylaminopurine at 8 µM), KIN4 (Kinetin at 4 µM) and TDZ1 (Thidiazuron at 1 µM). [3] Plant sizes: Large (12-16 mm); Medium (8-12 mm); Small (4-8 mm). [4] TE= Type of explant. [5]Values followed by different letters are statistically different for p =0.05 according to Student-Newman-Keuls.

On the other hand, it was found that the rooting capacity of the explants was closely related to the type of explant used, as apical and epicotyl explants showed a much higher response percentage (around 50-52 %) compared to central disc (13%) and hypocotyl (22%) explants (**Table 6**). Rooting in epicotyl explants would be expected, as root emergence is a natural process that spontaneously occurs in many species of cacti in response to damage or loss in the basal section of plants or their roots [51]. In contrast, the lower average rooting of hypocotyl explants could be due to their unnatural position in the medium. By contrast, rooting in apical explants was significantly higher than that observed in central discs, despite being similar-sized explants placed in a natural position. This confirms that plant dissection often alters the proportion of endogenous hormones in these structures, favoring the establishment of high auxin/cytokinin ratios in apices and consequently promoting root production in these types of explants [52,53]. On the contrary, this hormonal alteration does not occur in central discs, leading to a significant reduction in rooting events (**Table 6**).

Furthermore, rooting ability also appears to be related to the percentage of coloration of the explants used. In fact, explants from fully chlorophyllous plants rooted in a higher proportion than variegated ones (**Table 6**). Once again, these results seem to be associated with the greater vigor of chlorophyllous plants compared to plants with different degree of variegation. Finally, when studying the rooting percentages in detail for each of the test groups, it could be determined that rooting events for *Gymnocalycium* occur more frequently in the absence of cytokinins and when using apices or epicotyls from fully chlorophyllous plants (**Table 7**). On the contrary, the presence of TDZ1 blocks root emergence and limits rhizogenesis (**Table 7**).

Table 7. Percentage of rooting during the first three months per combination of factors.

Plant size[1]	Type of explant	% Color	TREATMENT [2]											
			BAP8			KIN4			TDZ1			CONTROL		
			1st Month	2nd Month	3rd Month	1st Month	2nd Month	3rd Month	1st Month	2nd Month	3rd Month	1st Month	2nd Month	3rd Month
Large	Apical	0	-	-	-	-	-	-	-	-	-	100.00	100.00	100.00
		25	16.67	66.67	66.67	66.67	66.67	66.67	0.00	0.00	0.00	-	-	-
		50	100.00	100.00	100.00	100.00	100.00	100.00	-	-	-	-	-	-
		75	50.00	50.00	50.00	50.00	100.00	100.00	0.00	0.00	0.00	-	-	-
	Central Disc	0	-	-	-	-	-	-	0.00	0.00	0.00	14.29	28.57	50.00
		25	0.00	0.00	0.00	16.67	16.67	16.67	0.00	0.00	0.00	-	-	-
		50	0.00	0.00	0.00	50.00	50.00	50.00	0.00	0.00	0.00	-	-	-
		75	25.00	25.00	25.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
Medium	Apical	0	50.00	93.75	93.75	87.50	93.75	93.75	0.00	0.00	0.00	84.38	96.88	96.88
		25	50.00	80.00	80.00	90.00	90.00	90.00	10.00	10.00	10.00	-	-	-
		50	50.00	75.00	75.00	100.00	100.00	100.00	0.00	0.00	0.00	-	-	-
		75	28.57	28.57	28.57	42.86	42.86	42.86	0.00	0.00	0.00	-	-	-
		0	12.50	12.50	12.50	18.75	31.25	31.25	0.00	0.00	0.00	6.25	12.50	21.88

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	Central Disc	25	10.00	10.00	10.00	20.00	20.00	20.00	0.00	0.00	0.00	-	-	-
		50	0.00	0.00	0.00	25.00	50.00	50.00	0.00	0.00	0.00	-	-	-
		75	0.00	0.00	0.00	0.00	21.43	21.43	0.00	0.00	0.00	-	-	-
Small	Epicotyl	0	-	-	-	-	-	-	-	-	-	93.75	93.75	100.00
		25	100.00	100.00	100.00	0.00	100.00	100.00	0.00	0.00	0.00	-	-	-
		50	25.00	50.00	100.00	25.00	50.00	50.00	0.00	0.00	0.00	-	-	-
		75	12.50	50.00	87.50	62.50	75.00	87.50	0.00	0.00	0.00	-	-	-
		100	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
	Hypocotyl	0	-	-	-	-	-	-	-	-	-	6.25	6.25	6.25
		25	0.00	0.00	33.33	0.00	100.00	100.00	0.00	0.00	0.00	-	-	-
		50	0.00	0.00	50.00	0.00	0.00	25.00	0.00	0.00	0.00	-	-	-
		75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
		100	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
Total for treatment			21.71	33.55	38.82	36.67	46.67	48.00	0.68	0.68	0.68	49.19	55.65	61.29

(1) Plant sizes: Large (12-16 mm); Medium (8-12 mm); Small (4-8 mm). (2) Treatments: BAP8 (6-Benzylaminopurine at 8 µM), KIN4 (Kinetin at 4 µM) and TDZ1 (Thidiazuron at 1 µM).

2.3. Color Evaluation

A comprehensive evaluation of each individually activated areola was conducted to determine the productive capacity of variegated shoots, taking into account both the percentage of variegation in the starting plants and the tissue coloration observed in the areola itself. The total count of shoots exhibiting varying degrees of coloration, categorized based on the color of the original plant, is presented in **Table 8**. As expected, plants from the 0% and 100% groups were only able of activating G and C areolas, respectively. However, explants from partially variegated plants (25%, 50%, and 75%) had the ability to activate areolas from all three groups previously defined (G, M, or C), with results included in **Table 8**. Therefore, it was essential to determine if there was any correlation between the initial variegation percentage of the plants, the type of areola activated, and the degree of coloration of the obtained shoots.

Table 8. Number and type of the activated areolas and the percentage of coloration of the obtained shoots in relation to the percentage of coloration of the initial plants.

% color of the original plant	Nº of activated areolas	Color of activated areola(1)			Percentage of shoot coloration			
		C	M	G	0%	<50%	>50%	100%
0	368	0	0	368	368	0	0	0
25	177	31	57	89	112	18	14	33
50	107	22	44	41	55	21	10	21
75	217	94	79	44	77	18	21	101
100	5	3	2	0	0	2	1	2
Total	874	150	182	542	612	59	46	157

(1) Color of activated areola: "G", green areolas; "M", mixed areolas; and "C", colored areolas.

Canonical correlation analyses revealed a strong correlation between the initial percentage of plant coloration and the type of activated areola, as well as between the initial percentage of plant coloration and the degree of coloration of the obtained shoots (**Table 9**). Furthermore, a strong correlation was also observed between the type of activated areola and the final coloration degree of the shoots (**Table 9**). Conversely, no correlation was detected between the applied hormonal treatment and the type of activated areola or the coloration degree of the obtained shoots (**Table 9**), indicating that the hormonal treatment may not have a significant effect on obtaining shoots with different color gradients.

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Table 9. Canonical correlation between evaluated factors and their corresponding significance values (*p*-value) for each combination.

Interactions	*p*-Value
Color percentage of the initial plant * Color of the activated areolas	0.000
Color percentage of the initial plant * Color percentage of the obtained shoots	0.000
Color of the activated areolas * Color percentage of the obtained shoots	0.000
Hormonal treatment * Color of the activated areolas	0.133
Hormonal treatment * Color percentage of the obtained shoots	0.766

The table shows the estimated correlations between each set of canonical variables. *P*-values lower than p =0.05 evidence a statistically significant correlation with a 95.0% confidence level.

In **Figure 4** it can be observed that, as the initial plants exhibit a higher degree of variegation, the number of colored areolas activated also increases. It appears that there is no limitation on the activation of M or C areolas compared to G ones. Therefore, the activation process seems to be affected by factors unrelated to the color of the areola, and the activation of a higher number of colored areolas in plants with a higher percentage of initial variegation may be just randomly. In this context, as the percentage of variegation in the initial plants increases, the likelihood of obtaining colored shoots also increases (**Figure 5**).

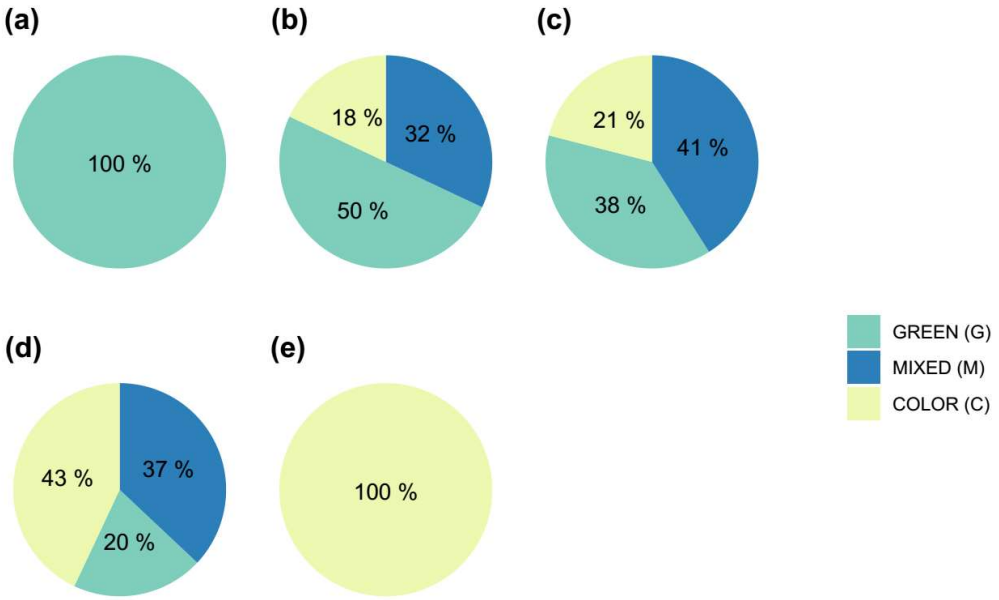

Figure 4. Percentage of activated areoles of each type (green = G, mixed = M or color = C) as a function of the initial coloration of the plants. **(a)** Green plants without variegation; **(b)** plants with 25% of variegation **(c)** plants with 50% of variegation, **(d)** plants with 75% of variegation and **(e)** plants completely colored (100% variegation).

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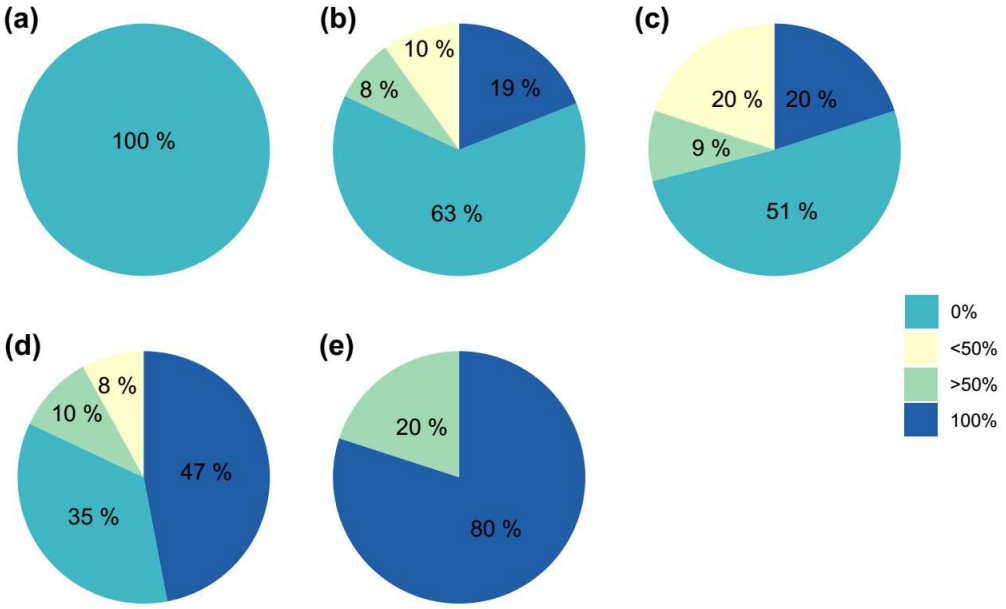

Figure 5. Percentage of obtained shoots with different degrees of variegation (0%, less than 50%, more than 50% and totally variegated, 100%) with respect to the percentage of variegation of the initial plants. **(a)** Green plants without variegation; **(b)** plants with 25% of variegation **(c)** plants with 50% of variegation, **(d)** plants with 75% of variegation and **(e)** plants completely colored (100% variegation).

Since green areolas from the control group only resulted in chlorophyllous shoots and contributed to a remarkable percentage of the evaluated areolas (42%), an individualized evaluation of each type of independent areola and its corresponding generated shoot was conducted to avoid biases in the results, using only those areolas from plants with some initial coloration (**Table 10**). The representation of the results showed that the coloration of the shoot was determined by the presence of chlorophyllous or variegated tissue in the areola (**Figure 6**). Therefore, green areolas (regardless of the variegation proportion of the initial plant) produced 97% green shoots, while colored areolas led to 98% colored plants, and from mixed areolas, 41% shoots were green and 59% shoots showed a range of degrees of coloration (**Figure 6**).

Table 10. Number of shoots obtained from the initial variegated plants as a function of the type of activated areola. Number of shoots obtained from areoles derived from the initial variegated plants classified according to the type of areola (shoots from the activated areoles of the initial chlorophyllic plants are not included).

Type of areola	Coloration of shoots [1]			
	S0	S1	S2	S3
Color (C)	3	4	15	129
Mixed (M)	74	48	31	28
Green (G)	166	5	1	0
Total	243	57	47	157
Total of shoots	504			

[1] Coloring percentage of shoots: group S0 = green shots without variegation; group S1 = shoots with a color percentage below 50%; group S2 = shoots with a color percentage above 50%; and group S3 = completely colored, without chlorophyllous tissue.

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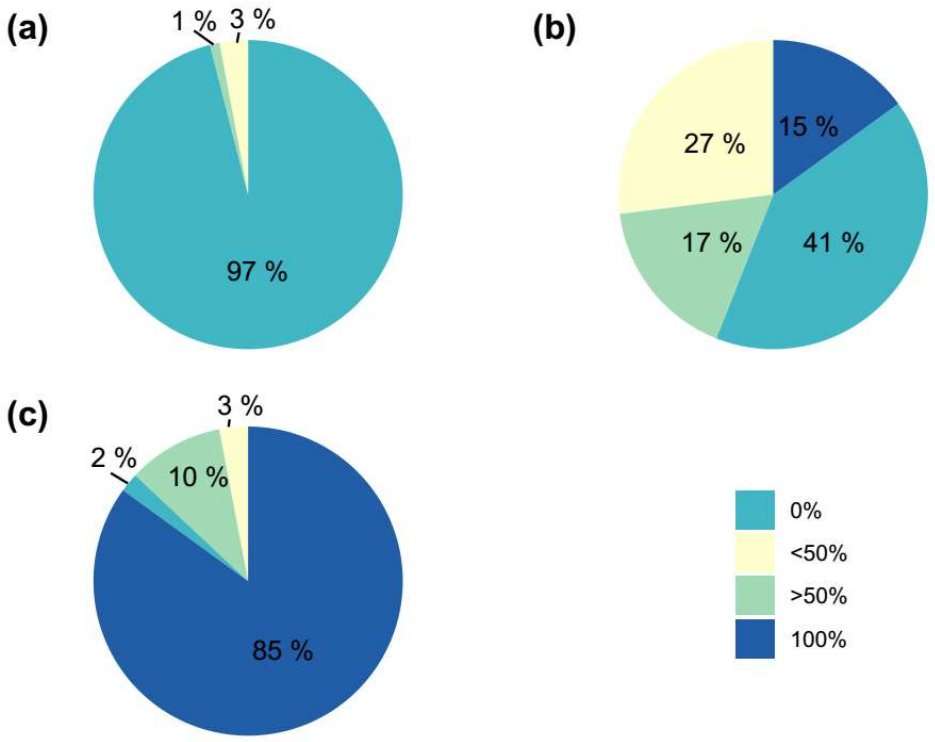

Figure 6. Percentage of shoots obtained with different degrees of variegation (0%, less than 50%, more than 50% and totally variegated, 100%) with respect to the coloration of the starting areoles (**(a)** - Green; **(b)** - Mixed; **(c)** - Color).

These results may indicate the occurrence of somatic mosaicism in the areolar tissue. Therefore, at least two different cellular lines (chlorophyllous and variegated) would converge in the buds, being capable of generating shoots with varying degrees of variegation. The proportion of these cellular lines and their distribution in the bud would determine the greater or lesser variegation of the obtained shoots (**Figure 7**) [38,54,55]. These findings are highly interesting in a commercial context, as it could lead to selecting the type of areola to activate based on the coloration of shoots desired, optimizing large-scale production processes. Thus, the use of central discs would optimize propagation of colored *Gymnocalycium*, in comparison to apices, where selecting areolas would be more difficult due to their smaller size and less inter-areolar space.

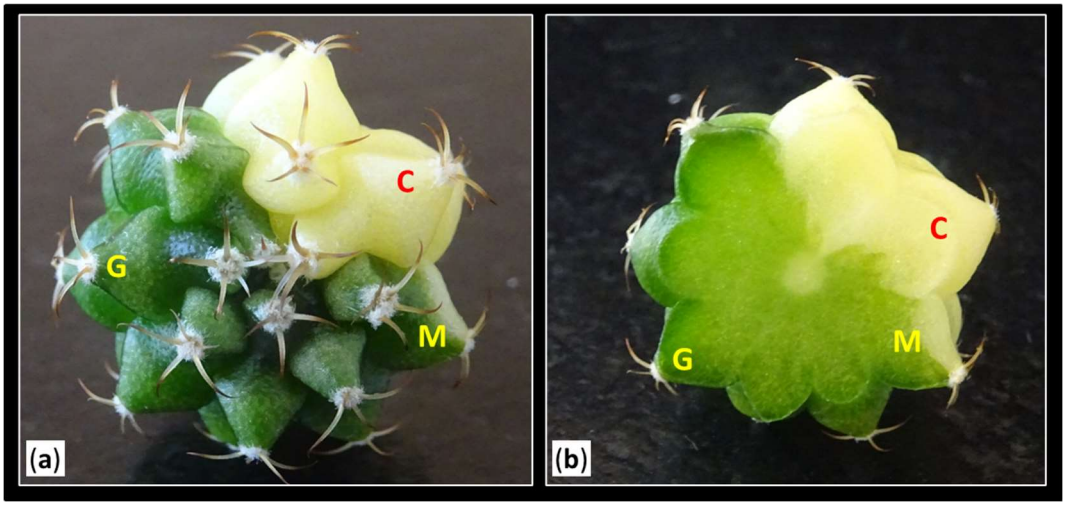

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Figure 7. Classification of the types of areolas according to their coloration. **(a)** View of the apical part of the plant. **(b)** Transverse section of a variegated plant. Letters indicates: "G", green areolas (where both the mamilla and areola were completely green); "M", mixed areolas (showing a combination of chlorophyllous and variegated tissue in both the mamilla and areola); and "C", colored areolas (where both the mamilla and areola were fully colored).

Additionally, shoots with new different colorations and color patterns were obtained in this trial (**Figure 8**), demonstrating that it is possible to produce color variants from plants that appear to have similar variegation. This fact highlights the complexity of the underlying mechanisms involved in the structuring and development of new shoots in variegated plants. Therefore, having a deep understanding on how various cell lines related to the appearance of new shoots with different colorations or color patterns can be activated, would be essential to carry out breeding programs aimed at obtaining new cultivars with different colorations.

Figure 8. Varied shoots with different colorations and different color patterns observed in the trial.

Moreover, fully or partially variegated variants can be of great commercial interest. In fact, many cultivars of *Gymnocalycium* with different colors (Hybotan, Seolhong, Damdan, Hwangweol, etc...), or the yellow peanut cactus (*Chamaecereus silvestrii* f. Lutea), have gained significant market relevance in recent decades [9]. According to the obtained results, propagation processes of forms or cultivars already in circulation could be optimized through the selection of explants with fully colored areolas. Furthermore, this protocol could be adapted for other variegated cactus species with potential in the wholesale market.

On the other hand, there is a significant market associated with cacti for hobbists, where customers are willing to pay higher prices for plants with distinctive and particular characteristics. Typically, variegations are among the aspects that collectors value. However, fully variegated plants (unable to survive on their own roots) usually do not appeal to this group, given the need for grafting to sustain them. The results obtained show that selecting mixed areolas as starting explants in a micropropagation protocol would allow for obtaining a very significant percentage of shoots with partial variegation (44% in this trial). These shoots could be isolated, rooted, and acclimatized, making their *ex vitro* development entirely viable. This would meet the expectations of collectors, opening up a new market niche associated with colored cacti.

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3. Materials and Methods

3.1. Plant Material and Disinfection

Plants were obtained by *in vitro* sowing of *Gymnocalycium* cv. Fancy seeds, kindly donated by Cactusloft OE (Cullera, Valencia, Spain). This commercial hybrid developed by Cactusloft O.E. (Cullera, Valencia, Spain) originated from controlled crosses between *Gymnocalycium mihanovichii* and *Gymnocalycium fiedrichi* (Werdermann) Pažout, resulting in progenies with diverse morphologies and colorations due to their broad genetic background. This circumstance gives *Gymnocalycium* cv. Fancy an enormous potential from a commercial point of view, given that plants with different degrees of variegation can be obtained and selected, and color variants with different morphologies can also be identified [22] (**Figure 2**). For their disinfection, seeds were treated under aseptic conditions in a laminar flow cabinet (model AH-100, Telstar, Terrassa, Spain) for 1 min in 70% ethanol (v/v), continued by 25 min in 15% domestic bleach solution (v/v; 4% sodium hypochlorite) supplemented with 0.08% of the surfactant Tween-20 (v/v). Finally, seeds were rinsed 3 times in distilled sterilized water before sowing.

3.2. In Vitro Establishment and Culture Conditions

Murashige and Skoog (MS) basal media (Duchefa Biochemie, Haarlem, Netherlands)[56] at half strength (1/2MS, 2.2 g L^{-1}) supplemented with 15 g L^{-1} of sucrose (Sigma-Aldrich, Missouri, USA) and 7 g L^{-1} of bacteriological agar (Duchefa Biochemie, Haarlem, Netherlands) was used as a sowing media. pH was adjusted to 5.7 before autoclaving at 120ºC for 20 min [57]. Disinfected seeds were sown into sterile plastic disposable Petri dishes containing 20 seeds each. Seedlings developed under *in vitro* conditions inside a growth room at 26±2ºC on shelves with a 16 h light / 8 h dark photoperiod and photosynthetic photon flux of 50 $molm^{-2}$ s^{-1} for 8 months. Seedlings were subcultured monthly to a fresh media.

3.3. Induction and Tissue Culture Conditions

With the aim of assessing the morphogenic potential of variegated seedlings of *Gymnocalycium* cv. Fancy with different degree of coloration, three specific concentration of cytokinins (Duchefa Biochemie Company, RV Haarlem, The Netherlands) that generated responses in chlorophyllous plants in previous works [22] were studied: 6-Benzylaminopurine 8µM (BAP8), Kinetin 4 µM (KIN4) and Thidiazuron 1µM (TDZ1).

The explants were placed on a culture 1/2MS media (2.2 g L-1), supplemented with sucrose (15 g L-1), agar (7 g L-1) and each of the three cytokinins (BAP8, KIN4 and TDZ1), adjusting the pH to 5.7, to activate their induction. Besides, a control group in absence of PGRs was included for each plant size and each type of explant. Furthermore, the explants were placed maximizing the contact between the sectioned tissue and the culture medium. The culture in the induction medium lasted for two months. A subculture was performed after the first four weeks to ensure that the hormone concentration was constant throughout the induction period. After the induction period, explants were subcultured to the initial basal 1/2MS media at pH 5.7 in absence of PGRs.

3.4. Experimental Design

After an 8 month period of *in vitro* growth, a total of 180 plants were selected and classified depending on their initial size (**Table 11**). From medium and large-sized plants, two types of explants were obtained: apical and central disc, that were in turn sectioned into two halves, i.e. four explants were obtained for each medium or large-sized plant. From small-sized plants, epicotyl and hypocotyl were obtained, through cutting transversally the seedlings into two parts. Roots were completely removed in all cases. Therefore, four different types of explants were evaluated in this study: apical explants, central disc explants, epicotyls and hypocotyls (**Table 11**).

Plants were visually classified into four groups depending on the proportion of coloration observed (25%, 50%, 75% and 100%). Considering their initial sizes, plants were randomly distributed

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in each group before obtaining the explants (**Table 11**). Fully colored plants were only included in the small-sized group, as their lack of chlorophyll provoked sizes < 8 mm of diameter. Furthermore, plants fully covered by chlorophyll (i.e. 0% color) were also included as a different control groups (**Figure 9**): (a) on one hand, medium-sized plants subjected to the presence of PGRs and, (b) on the other hand, plants of all evaluated sizes (small, medium and large-sized) in absence of PGRs (**Table 11**).

Table 11. Experimental design of the trial.

Plant size	% Color	Nº plants	N° of explants evaluated [1]				Treatment [2]			
			A	CD	EP	HYP	BAP8	KIN4	TDZ1	CONTROL
Large (12-16 mm)	0	7	14	14	-	-	-	-	-	28
	25	9	18	18	-	-	12	12	12	-
	50	2	4	4	-	-	4	4	-	-
	75	6	12	12	-	-	8	8	8	-
Medium (8-12 mm)	0	40	80	80	-	-	32	32	32	64
	25	15	30	30	-	-	20	20	20	-
	50	7	14	14	-	-	8	8	12	-
	75	21	42	42	-	-	28	28	28	-
Small (4-12 mm)	0	16	-	-	16	16	-	-	-	32
	25	7	-	-	7	7	6	4	4	-
	50	12	-	-	12	12	8	8	8	-
	75	24	-	-	24	24	16	16	16	-
	100	14	-	-	14	42	10	10	8	-
Total for condition			214	214	73	73	152	150	148	124
Total trial		180	574				574			

[1] Type of explant: A (apical), CD (central disc), EP (epicotyl), HYP (hypocoty) . [2] Treatments: BAP8 (6-Benzylaminopurine at 8 µM), KIN4 (Kinetin at 4 µM) and TDZ1 (Thidiazuron at 1 µM).

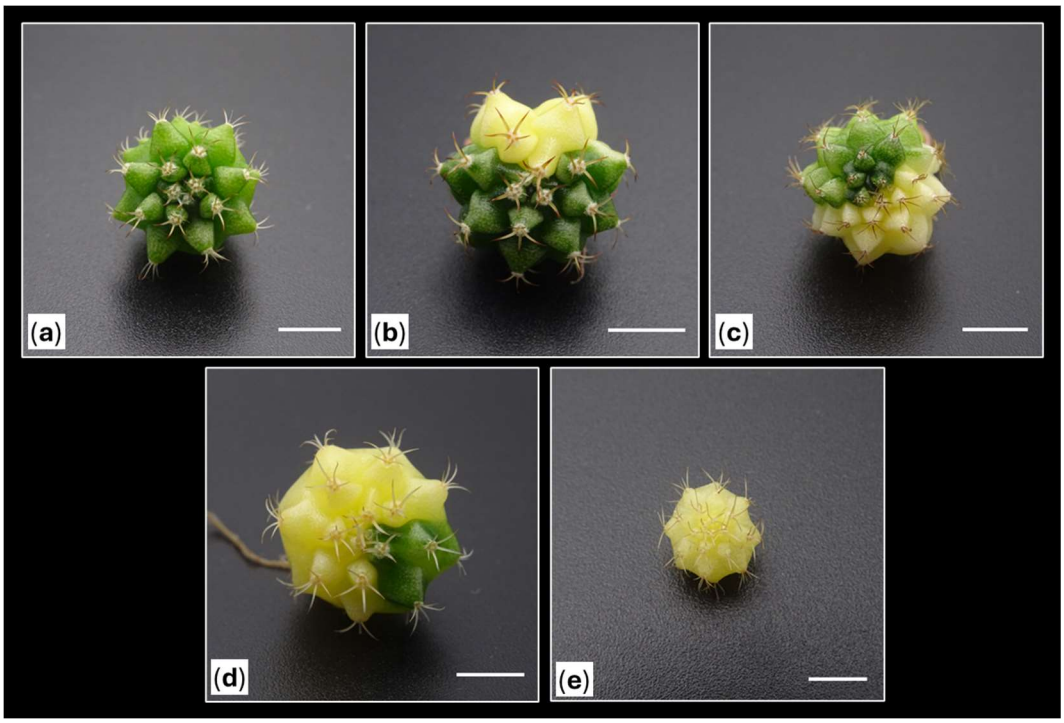

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Figure 9. Initial plant variegation percentage classification system: **(a)** completely chlorophyll plant (no variegation, 0%), **(b)** plants with more chlorophyll than nonchlorophyll tissue (25% variegation), **(c)** plants with equal proportion of chlorophyll and nonchlorophyll tissue (50% variegation), **(d)** plants with more than 50% nonchlorophyll tissue (75% variegation), and **(e)** plants with completely nonchlorophyll tissue (100% variegation). White bars = 10 mm.

A total of five 574 explants from plants with different color percentages, including 214 of each apical explants and central disc explants and 73 of each epicotyl and hipocotyl explants, were evaluated during the experiment (**Table 11**). The explants were analyzed considering the degree of variegation and size of the original plant for each of the hormonal treatments and the control group. They were distributed in groups of four explants per Petri dish for their evaluation.

The percentage of activated explants was calculated as the number of explants that showed some type of response during the assay, either organogenic or callogenic, relative to the total number of explants (**Table 1**). The appearance of shoots and the formation of calluses were noticed monthly for 5 months, only from those explants that responded to the treatments. Considering the different number of areolas present in each one of the starting explants (**Table 4**), the results were interpreted taking into account both: (i) productivity (i.e. total number of shoots per explant) and (ii) efficiency (i.e. ratio of the activated areoles with respect to the total of areoles of each explant). The emission of roots was also recorded in terms of percentage during the first three months.

3.4.1. Areole Evaluation

Using a magnifying glass (Kern OZO, 551), all activated areolas from colored plants were visually classified into three groups based on their coloration: green areolas "G" (where both the mamilla and areola were completely green), mixed areolas "M" (showing a combination of chlorophyllous and variegated tissue in both the mamilla and areola), and colored areolas "C" (where both the mamilla and areola were fully colored) (**Figure 7**). Chlorophyllous plants from the control groups were not included in this evaluation, since all the sprouts obtained in previous trials were totally green [22].

Subsequently, the obtained shoots were counted considering the percentage of coloration of the source plants and classified into four groups based on their final coloration: shoots completely green (without variegation, group "S0"), shoots with a coloration percentage below 50% (group "S1"), shoots with a coloration percentage above 50% (group "S2"), and completely colored shoots (without chlorophyllous tissue, group "S3") (**Figure 10**). The relationships between the percentage of coloration of the activated areolas and the coloration of the shoots obtained based on the percentage of color of the initial plants, as well as the coloration of the shoots obtained based on the coloration of the activated areolas, were evaluated.

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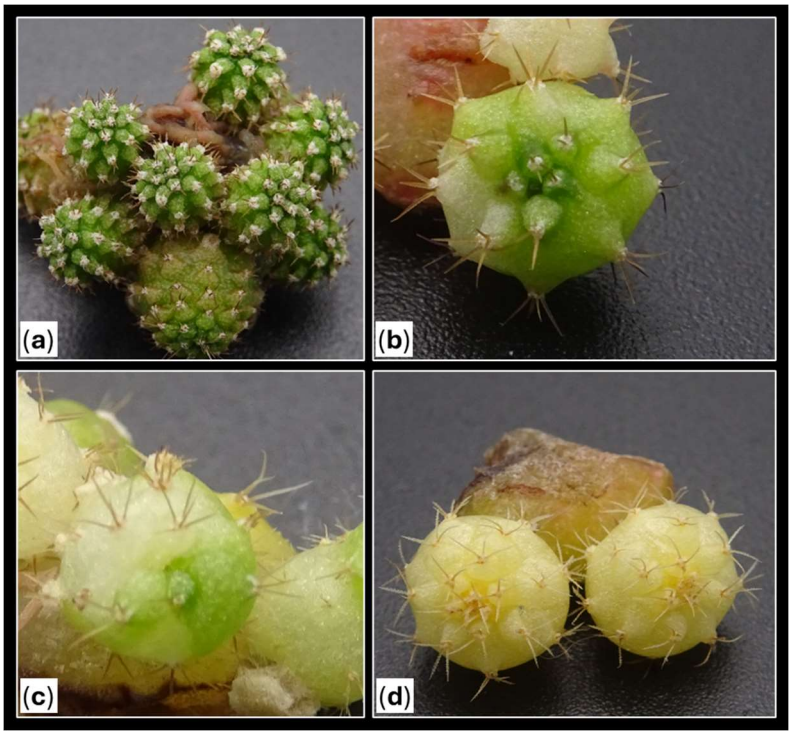

Figure 10. Grading system of the obtained shoots according to their final coloration. **(a)** completely green shoots (without variegation, group "**S0**"); **(b)** shoots with a percentage of coloration lower than 50% (group "**S1**"); **(c)** shoots with a percentage of coloration higher than 50% (group "**S2**"), and **(d)** completely colored shoots (without chlorophyll tissue, group "**S3**").

3.5. Statistical Analysis

In order to analize our data sets, multivariate ANOVA analysis was performed to check the effect of the different factors at a level of $p < 0.05$. The software used for performing this ANOVA analysis was Statgraphics Centurion XVIII (Statgraphics Technologies Inc., The Plains, Virginia, USA). The presence of the three different hormones (BAP8, KIN4 and TDZ1) in the culture media, the relevance of the initial size (small, medium and large-sized plants), the presence of diverse degree of variegation in the initial plants and the activation capacity of the various explants (apical explants, central disc explants, epicotyls and hipocotyls) were analized only in those explants that responded to some treatment.

Means differing significantly were compared using the Student-Newman-Keuls test with a probability level of the 5%. Transformation of the data was previously made to normalize the dataset using the following formulas:

- For numerical and absolute data, including shoot emission, callus production and averages.

$$\sqrt{Y + \frac{1}{2}}$$

- For percentages and efficiency values, including rooting capacity.

$$arcsin\sqrt{\frac{percentage}{100}}$$

The linear combinations between the initial color of the plants, the color of the activated areolas, the color of the shoots obtained and the hormones used in the trial were studied through canonical correlation analysis, establishing a confidence level of 95%. Statgraphics Centurion XVIII (Statgraphics Technologies Inc., The Plains, Virginia, USA) was also used for these analysis.

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4. Conclusions

In this work, a specific micropropagation protocol focused on obtaining shoots with varying degrees of variegation in *Gymnocalycium* cv. Fancy plants has been successfully optimized. The use of central disc explants in the presence of TDZ1 in the culture medium yielded the best results in terms of initial explant activation, shoot productivity and efficiency related to areolar activation per explant. Furthermore, it was observed that the coloration percentage of the starting plants (excluding completely achlorophyllous plants) did not limit the response capacity of the evaluated explants. Hence, a strong correlation exists between the initial variegation percentage of the plants and the type of activated areola.

Additionally, the type of activated areola correlated with the color percentage of the obtained shoots, highlighting a situation of somatic mosaicism in the areolas that determines the variegation percentage of the final shoot. These results allow for adjusting and optimizing propagation protocols to obtain plants with different variegation proportions based on commercial objectives. So that, for obtaining fully variegated shoots, colored areolas would be selected, while for obtaining shoots with partial variegation, explants carrying mixed areolas would be chosen.

Therefore, this protocol is extremely valuable from a commercial standpoint as it enables control and prediction of the coloration of the obtained plants, thus allowing them to be directed towards high-impact markets (wholesale or collector). Consequently, achieving greater efficiency and resource optimization in commercial plant production processes would be possible.

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