# Effects of the in vitro behavior of micropropagated plants on the stability of variegation in Yucca gloriosa, Phormium tenax, and Cordyline australis cultivars

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# *In vitro* propagation behavior influences the variegation stability of *Yucca gloriosa*, *Phormium tenax* and *Cordyline australis* cultivars

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## Key words

Ornamental plant, Variegation, Micropropagation, Histology, Chimera, True-to-type

## Abbreviations

AdM: Adventitious Bud Meristem; AxM: Axillary Bud Meristem; BAP: 6-benzylaminopurine; BSM: Basal Solution Medium; CaPP: *Cordyline australis* 'Pink Passion'; IAA: Indole-3-acetic acid; NPA: N-1-naphthylphthalamidic acid; OT: Off-Type; PGR: Plant Growth Regulators; PtJE: *Phormium tenax* 'Jessie'; SAM: Shoot Apical Meristem; SMM: Shoot Multiplication Medium; TTT: True-to-Type; WT: Wild-Type; YgVAR: *Yucca gloriosa* 'Variegata'.

# Highlights

- Individualized plant monitoring revealed large differences in the multiplication rates and phenotypes obtained from true-to-type variegated cultivars
- Histological observations of meristems revealed that each species presented major differences during *in vitro* development and multiplication behavior
- Laser scanning confocal microscopy of leaf tissues revealed chimeric layers with different contributions to leaf development
- All cultivars are periclinal chimeras whose variegation stability depends on their propensity to propagate by adventitious meristems
- Production potentials of these variegated cultivars are discussed from an industrial point of view

# Abstract

Cultivars with variegated foliage could be difficult to obtain in large-scale production of true-to-type plants. To determine the micropropagation potentialities of ornamental variegated monocotyledons in an industrial and commercial context, to define the stability of their variegated phenotypes, and to understand how and when off-type plants appear, the shoot tips of a variegated cultivar of three different species : *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and *Cordyline australis* 'Pink Passion' were introduced *in vitro* for propagation. The propagation rates and the variegation stability were estimated by separately monitoring each plant obtained during the several successive subcultures. During the *in vitro* process, samples were periodically fixed and studied by staining histological sections of the shoot tips. Leaf tissues were studied by light and confocal microscopy.

After three-four subcultures, the multiplication rates obtained were stabilized at around 2.5 to 3 for *Yucca*, 1.4 to 1.7 for *Phormium,* and 1.2 to 1.7 for *Cordyline*. The number of off-type plants obtained after six multiplication cycles was around 10% for *Yucca*, 25% for *Phormium* and 60% for *Cordyline*. The histology study showed that the axillary bud meristems (AxM) are totally (*Yucca* and *Cordyline*) or partially (*Phormium*) repressed by shoot apical meristem (SAM) in PGR-free medium. In the presence of BAP or BAP and NPA, only AxM were observed for *Yucca*, *Phormium* mainly developed AxM and some adventitious bud meristems (AdM), and *Cordyline* developed both AxM and AdM. The leaf section observations revealed that the three variegated cultivars turned out to be three periclinal chimeras.

This histological study carried out on these cultivars highlights major differences in the development and *in vitro* multiplication behavior of three different genera belonging to the order Asparagales (clade monocotyledons). The variegation stability of these chimeric cultivars depends on their propensity to propagate by adventitious meristems.

# 1. Introduction

Variegation is defined as the presence of multiple colors on leaves, flowers or stems, with regular or irregular patterns. On foliage, it provides an attractive and colorful visual appearance, an important trait for ornamental plants (Behe and Nelson, 1999; Henny and Chen, 2003; Li et al., 2007). An understanding of the determinism of the variegation as well as of its heritability is therefore essential when selecting new cultivars (Cao et al., 2016). It could also help to control growing conditions that would lead to both the good expression of the variegation and to favorable propagation conditions that would ensure that the plantlets are true-to-type (Vladimirova et al., 1997; De Keyser et al., 2019).

Leaf variegation could be related to two main origins: either a special tissue structure and/or pigment heterogeneity. Structural variegation (physical color caused by the optical properties of leaf anatomy) includes the phenomenon of blistered leaves (Sheue et al., 2012) and the juxtaposition of different epidermal cells (Chen et al., 2017; Pao et al., 2020). Pigment-related variegation (chemical color) is mainly due to variations in chlorophyll content, whereas carotenoids, flavonoids and anthocyanins also contribute to obtaining multicolored leaves (Ahmed et al., 2004; Klancnik et al., 2016).

These differences in pigmentation can have many causes: differential gene expression depending on the location on the leaf (Marcotrigiano, 1997), viruses that cause non-uniform chlorosis (Fulton, 1964; Marcotrigiano, 1997), plastome mutations (Wildman, 1973; Tilney-Bassett, 1975), mitochondrial genome mutations (Newton and Coe, 1986; Bonnett et al., 1993), etc. However, the variegation linked to a deficiency in chloroplast development is one of the most common and studied variegation mechanisms (Fisher, 1986; Aluru et al., 2001; Sakamoto, 2003; Putarjunan et al., 2013; Tsai et al., 2017; Cao et al., 2018; Li et al., 2019). This phenomenon can occur in mutants with identical genotypes in the green and chlorotic sectors, providing interesting cases for epigenetic regulation studies (Cocciolone and Cone, 1993; Wang et al., 2016; Duarte-Aké et al., 2016) or for the investigation of chloroplast biogenesis pathways (Sakamoto, 2003; Putarjunan et al., 2013). However, leaf variegation can also occur when the cells of the two distinguishable color sectors have distinct genotypes, a phenomenon referred to as genetic mosaicism (Marcotrigiano, 1997). Periclinal chimeras are a special case of genetic

mosaicism where an entire layer of the shoot apical meristem is genetically distinct from the others (Frank and Chitwood, 2016). The three layers (L1-L2-L3) of the meristem are maintained during plant development and contribute to the formation of the different organ tissues. In leaves, this leads to the phenomenon of variegation when a layer is genetically different in terms of anthocyanin synthesis or chloroplast biogenesis (Stewart and Dermen, 1979; Marcotrigiano, 1997; Frank and Chitwood, 2016).

Tissue culture techniques are useful for the rapid vegetative propagation of a wide range of ornamental plants. However, the value of these techniques depends on the efficiency and the reliability of producing true clones of the original genotype. In tissue culture, the plants produced can have several origins and modes of development: (1) axillary branching, originating from preformed meristems; (2) direct adventitious *organogenesis* (i.e., not from preformed meristems but with shoots arising directly from plant cells in unusual locations); (3) indirect adventitious *organogenesis* (i.e., shoot regeneration via a callus phase); (4) direct somatic *embryogenesis*; *or* (5) indirect somatic *embryogenesis* (George and Debergh, 2008; Phillips and Garda, 2019). However, it appears that adventitious *organogenesis* and indirect *embryogenesis* systems tend to accumulate more spontaneous mutations compared to direct somatic *embryogenesis*, but that the most stable system with the fewest variations is axillary branching (Chu, 1992; Vazquez, 2001; George and Debergh, 2008; Zayova et al., 2010; Phillips and Garda, 2019).

In 1981, Larkin and Scowkraft proposed a general term, “somaclonal variation”, for plant variants derived from tissue cultures, with different mechanisms involved: hyper/hypomethylation of DNA, changes in chromosome number, chromosomal rearrangements, and DNA base deletion/substitution due to oxidative stresses during tissue culture procedures (Krishna et al., 2016).

In the case of variegated plants, true-to-type plants are commonly obtained by tissue culture when variegation is due to differential gene expression, e.g., on *Aglaonema* (Yeh et al., 2007), *Codiaeum variegatum* (Radice, 2000 and 2010) and *Dracaena surculosa* (Liu et al., 2010). In contrast, for variegation due to epigenetic phenomena, the new plants obtained may be very heterogeneous, e.g., on *Clivia miniata* (Wang et al., 2016) and *Agave angustifolia* (Duarte-Aké et al., 2016). For the specific case of periclinal chimeras, the mode of multiplication has a very pronounced effect on the maintenance of the variegated character. A high proportion of true-to-type plants are obtained by axillary branching (preformed meristems), while the adventitious meristems from *organogenesis* modes reveal very high

111 frequencies of off-type plants in many genera like *Yucca* (Pierik and Steegmans, 1983), *Ajuga reptans*
112 (Lineberger and Wanstreet, 1983), *Rubus* (McPheeters and Skirvin, 1983), *Saintpaulia* (Lineberger and
113 Druckenbrod, 1985), *Nicotiana* (Marcotrigiano, 1986), *Rhododendron* (Pogany and Lineberger, 1990),
114 *Fragaria* (Marcotrigiano et al., 1997) and *Liriope* (Amory and Gill, 1999).
115 The aim of this study is to assess the feasibility of micropropagating variegated cultivars of three
116 different species of ornamental monocotyledons in an industrial and commercial context: *Yucca gloriosa*
117 'Variegata', *Cordyline australis* 'Pink Passion' (both *Asparagaceae*) and *Phormium tenax* 'Jessie'
118 (*Xanthorrhoeaceae*).
119 Shoot tips of each cultivar were micropropagated via several successive subcultures using three different
120 multiplication media, with the objective of stimulating axillary branching. An original aspect of this
121 work is that the multiplication rates and leaf variegation were checked for each *in vitro* plant through
122 the successive subcultures, making it possible to characterize the development of each genus, and the
123 precise evolution of variegation of each cultivar. At the same time, a laser scanning confocal microscopy
124 was performed to determine the developmental mode of the newly formed shoots. A histological
125 analysis of the leaf tissues of different phenotypes was carried out on each species in order to
126 characterize the variegation. All these data should help to better understand the mechanisms behind the
127 variegations of our cultivars, to define their stability and, finally, to determine the best conditions for
128 the production of true-to-type plants.

# 2. Materials and methods

## 2.1. Plant material and growth conditions

A variegated cultivar was studied for each of the three selected genera: *Yucca gloriosa* 'Variegata' (YgVAR) with green leaves and thin white edges; *Phormium tenax* 'Jessie' (PtJE) with green leaves and a pink stripe in the center; and *Cordyline australis* 'Pink Passion' (CaPP) with purple leaves and pink edges (Fig. 1). YgVAR and CaPP plants were issued from *in vitro*-propagated material, whereas PtJE plants were the result of *in vivo* vegetative propagation. For comparative purposes, *Phormium tenax* and *Cordyline australis* wild type plants (PtWT and CaWT respectively) with uniform green leaves were grown from seed. With no available *Yucca gloriosa* wild-type plants, a closely related species, *Yucca filamentosa* wild-type (YfWT) was also used for comparative purposes, and grown from seed.
Plants were transplanted in 500 ml plastic pots containing a horticultural substrate (25% pine bark, 50% Baltic peat and 25% coconut fiber), and grown under plastic tunnels in frost-free conditions.

## 2.2. Micropropagation from shoot tips

For *Yucca gloriosa* 'Variegata' and *Phormium tenax* 'Jessie', shoot tips of 1 cm were excised from one-year-old stock plants growing in plastic tunnels after *in vitro* and *in vivo* propagation, respectively. Explants were rinsed in sterile water, disinfected by soaking in 70% ethanol for 5 min and 5% sodium hypochlorite (NaClO) for 15 min. After rinsing three times in sterile water, the outer leaves were carefully removed and the explants (207 and 229 shoot tips for YgVAR and PtJE, respectively) were introduced *in vitro* by placing them in sterilized plastic tube cultures with 10 ml of basal solution medium (BSM) composed of Murashige and Skoog medium without plant growth regulators (Murashige and Skoog, 1962), supplemented with 2.5% sucrose and 0.6% agar powder (Agar HP697, Kalys, Bernin, France) for which the pH was adjusted to 5.7 before autoclaving. For *Cordyline australis* 'Pink Passion', rooted shoots were purchased from a private laboratory of tissue culture and directly introduced on BSM

after root elimination (240 shoot tips). For the introduction step, cultures were conducted under a 16-h photoperiod provided by cool-white fluorescent tubes (Sylvania, Luxline Plus, Daylight-type, Germany) at 25 µmol $m^{-2}$ $s^{-1}$ at 26°C.

After 3 weeks, healthy shoot tips were transferred to shoot multiplication medium (SMM) based on BSM, with three different plant growth regulator (PGR) combinations: (1) 6-benzylaminopurine (BAP), referred to as BAP medium in this study; (2) BAP and 1 µM of N-1-naphthylphthalamidic acid (an inhibitor of auxin transport; Teale and Palme, 2018), referred to as NPA medium; and (3) without plant growth regulators for a control condition, referred to as Control medium (Fig. S1). BAP was used at 66.59 µM (*Yucca)* and 4.44 µM (*Cordyline* and *Phormium),* depending on the laboratory experience. Cultures were then conducted under a 16-h photoperiod provided by cool-white fluorescent tubes adjusted to 50 µmol $m^{-2}$ $s^{-1}$ at 26°C. Shoot tips were subcultured and eventually divided every 3 weeks (*Yucca*) or 4 weeks (*Cordyline* and *Phormium*). After three or six clonal subcultures (whose multiplication cycles were referred to as M1, M2, etc.), shoots were transferred to a shoot elongation step on BSM without plant growth regulators for one subculture (E1) for *Yucca*, and two (E1 and E2) for *Cordyline* and *Phormium*. Shoot tips were then transferred for rooting on BSM supplemented with 5.71 µM of IAA. After 3 weeks for rooting, plants were transplanted to a plug tray (composed of 80% cocopeat and 20% peat) before acclimatization in a greenhouse at a temperature above 20°C and manual humidity management. After 2 months, plants were transferred to plastic tunnels under frost-free conditions.

### 2.3. Histological analyses of meristematic areas

Histological analyses were performed on micropropagated *in vitro* plants every 10 days during the first two clonal subcultures and at the end of the 6$^{th}$ clonal cycle on SMM (Fig. S1). Samples were vacuum-fixed at 4°C for 72 h in 0.2 M phosphate buffer at pH 7.2, supplemented with 2% (v/v) paraformaldehyde, 1% (w/v) caffeine and 1% (v/v) glutaraldehyde, according to the modified Jouannic et al. (2011) protocol. Then, after a progressive dehydration in ethanol series from 50 to 100% (1 h for each one and 72 h for the last one at 100%), each sample was finally pre-impregnated in ethanol/resin

(v/v) for 96 h, then impregnated in resin (96 h) and finally embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrhrim, Germany). Longitudinal sections of 3 µm were made every 25 µm over the entire thickness of plants using a microtome (Leica RM2265, Wetzlar, Germany). Slides were double-stained with periodic acid-Schiff (PAS) reagent (Sigma–Aldrich, Lyon, France) for insoluble carbohydrate compound detection (Clark, 1984), and with Naphthol BlueBlack (NBB) for protein detection (Fisher, 1968). In the end, 1912, 2249 and 2052 sections were obtained and analyzed for *Yucca* 'Variegata', *Phormium* 'Jessie' and *Cordyline* 'Pink Passion', respectively, and microphotographs were taken with a stereomicroscope (SZX 16, Olympus, Tokyo, Japan).

### 2.4. Laser scanning confocal microscopy of leaf tissues

Leaf histological observations were made for *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and *Cordyline australis* 'Pink Passion' true-to-type plants (YgVAR TTT, PtJE TTT and CaPP TTT, respectively), and for off-type plants obtained from micropropagation (YgVAR OT, PtJE OT and CaPP OT, respectively), as well as for *Yucca filamentosa*, *Phormium tenax* and *Cordyline australis* wild-type plants grown from seeds or *in vivo* vegetative propagation in plastic tunnels and used as the control (YfWT, PtWT, CaWT). Cross-sections (25 µm) were obtained from fresh leaves embedded in 5% Low Melting Point Agarose, which melts at between 62 to 68°C (Amresco Low Melting Point Agarose, VWR Life Science), and cut using a vibratome (HM 650V, Microm, Walldorf, Germany). Microscope imaging was performed with a confocal laser scanning microscope (CLSM, Nikon Instruments, Melville, NY, USA) using a 10 or 20x 0.7 glycerol immersion lens. Excitation was provided by lasers at 405, 561 and 638 nm, with emissions captured from 425 to 475 nm (autofluorescence of lignin and cuticle), 570 to 620 nm (anthocyanin autofluorescence) and 662 to 737 nm (chlorophyll autofluorescence), respectively. Transmitted light images were routinely recorded with bright-field optics. Pseudocolor images could be generated by combining blue (405 nm), yellow (561 nm) and red (638 nm) transmitted light images with laser power adjusted so that the background transmitted light was even.

### 2.5. Phenotypic data and statistical analysis

212

213 During the micropropagation process, secondary shoots emerging from a shoot tip were excised at each
214 subculture, identified, observed and transferred to a new individual plastic tube in order to follow their
215 development. The formation of each new shoot was scored from SG1 to SG8, where SGx corresponds
216 to a shoot generated during the multiplication subculture x (Mx, i.e., SG3 and SG6, for new shoots
217 generated during cycles M3 and M6, respectively). SG0 corresponds to shoots initially introduced *in*
218 *vitro* on BSM without PGR, before transfer to the multiplication media for clonal subcultures M1 to M6
219 (see Fig. S1).
220 The multiplication rate was noted for each *in vitro* plant at each subculture as the number of
221 individualized shoots obtained per each cultivated shoot tip. The phenotypic conformity of the leaf
222 variegation for each *in vitro* plant was observed and noted according to the phenotypic characteristics
223 previously mentioned. The phenotype of the new shoots (daughter plants, e.g., SGx+1) was observed at
224 the end of the micropropagation process, after the elongation phase for *Yucca* 'Variegata' and *Cordyline*
225 'Pink Passion', and during the acclimatization phase for *Phormium* 'Jessie', and compared to that of the
226 shoots cultured on the previous subculture (mother plants, i.e., SGx in this example). The phenotypes of
227 a new shoot and of its mother plant were determined at the same time and associated *a posteriori.*
228 All of the healthy shoots were used at each subculture, except for YgVAR because, in order to limit the
229 number of plants, a part of the new shoots obtained after subcultures M4, M5 and M6 were randomly
230 selected and excluded from the subsequent subcultures.
231 After the 3$^{rd}$ multiplication subculture, a part of the shoots was transferred to the shoot elongation step
232 to evaluate the variegation. The rest was subcultured for three additional multiplication cycles (up to
233 M6), with, as a consequence, a reduction in the number of individuals after the 3rd subculture (see Fig.
234 S1).
235 Statistical analyses were performed with the ANOVA test and Tukey's multiple comparison tests
236 ($p=0.05$), or the Kruskal-Wallis test ($p=0.05$) when the number of individuals was too low. For the
237 phenotypic observations, bimodal phenotype data (i.e. 0 for true-to-type and 1 for off-type phenotype
238 respectively) were fitted in logistic models. Pearson's Chi-squared test with the Yates's continuity
239 correction ($p=0.05$), in R software (version 4.0.3), was also used.

# 3. Results

## 3.1. Multiplication rates during the *in vitro* micropropagation process

Shoots were micropropagated on three media (Control, BAP and NPA), and the global multiplication rate was defined for each clonal cycle, when shoots were subcultured for subsequent cycles (1 corresponding to "no multiplication", see "Tot. M1 to E2" in Table 1, and Fig. S2 for graphical data). For *Yucca gloriosa* 'Variegata', the shoots showed no multiplication at all on the control medium (Table 1A). On media with PGR (Fig. 2-A-B), the multiplication rate was slightly higher than 1 during M1 (1.13 and 1.28 for BAP and NPA, respectively) and was then multiplied by 2 during M2 (2.02 and 2.55 for BAP and NPA, respectively). For both media, the multiplication rate then stabilized between 2.5 and 3 from M3 to M6. The multiplication rate remained stable during the shoot elongation step E1 (Fig. 2 C, mean of 2.39 and 2.79 for BAP and NPA, respectively). For all of the cycles, i.e., for the average of all subcultures, the BAP medium led to a significantly lower multiplication rate than that of NPA, according to Tukey's HSD multiple comparison test (mean of 2.37 and 2.59 for BAP and NPA respectively).

For *Phormium* 'Jessie', a very low multiplication occurred on the control medium (1.05, the average of all of the cycles; Table 1B). With PGR (Fig. 2 E-F), no secondary shoot emerged during the first multiplication cycle (M1). The shoot tips then presented a multiplication with a maximum in M3 (mean of 1.93 and 2.16, respectively, for BAP and NPA). The multiplication rate then decreased and reached an equilibrium on M4 to M6 and on E1 (average of 1.61 and 1.66, respectively, for BAP and NPA). During the second elongation cycle (E2), no new shoots were formed, regardless of the SMM used before. For all of the cycles, medium with PGR had a significantly higher multiplication rate than the control, but with no differences between BAP and NPA (mean of 1.41 and 1.41for BAP and NPA, respectively).

For the cultivar *Cordyline australis* 'Pink Passion', like for YgVAR, the shoots showed no multiplication rate on the control medium during the eight successive subcultures (Table 1C). On media

with PGR (Fig. 2-I-J), the shoots showed a very low multiplication rate during M1 (1.00 and 1.02 for BAP and NPA, respectively). Then, from M2 to M6, the shoots presented low but stable rates (between 1.15 and 1.60, and between 1.24 and 1.72 for BAP and NPA, respectively). The first elongation cycle (E1) produced low multiplication rates (1.47 and 1.05 for BAP and NPA, respectively), but the second one (E2) gave higher rates (2.29 and 2.03 for BAP and NPA, respectively). For all of the cycles, Tukey's HSD multiple comparison tests show no difference between the BAP and NPA combination (1.41, 1.42 and 1.00 for BAP, NPA and Control, respectively).

In order to study the differences in *in vitro* behavior and development in these three botanical genera, the multiplication rate for each generation of shoots was determined through the various subcultures on the three different media (Table 1 and Fig. S2).

For YgVAR (Table 1A), for the first generations of shoots, SG0 to SG2, the multiplication rates strongly increased at each new cycle up to M4, and then stabilized or even decreased (except SG1). For the generations SG3 to SG6, the increase in the multiplication rates was lower and the cultures reached a multiplication equilibrium more quickly.

For PtJE (Table 1B), no secondary shoots elongated during M1 on either media, resulting in no SG1 plants. For each generation, the multiplication rate was low (less than 1.2) during its first subculture on SMM (1.00 to 1.28) (Table 1B) and then presented a peak between the cycles M3 and M6. The highest multiplication rates were for SG0 (with a maximum of 2.83 and 2.68 with BAP and NAP, respectively) and decreased according to the generations. After transfer to the elongation step, the multiplication rate increased during E1, except for SG0 (reaching an average of 2.15 for the SG2 to SG5 generations). No more multiplication occurred on E2.

For CaPP (Table 1C), the SG0 shoots presented no or few multiplications on M1 medium with PGR (1.00 and 1.02 for BAP and NPA respectively), and their multiplication rate remained low for every subsequent cycle (M2 to M6, with a maximum of 2.09). The shoots SG1 to SG6 had an even lower rate (except for SG3 during M5 with BAP), with an average of 1.09 and 1.21, for all of the cycles from M1 to M6, for BAP and NPA, respectively (Table 1C). During the first elongation step (E1), the multiplication rate remained low, whereas during E2, the SG2 to SG7 cycles presented multiplication

rates as high as 3.00. A high bacterial contamination explained some loss of plants, especially for SG3 after M6 on BAP medium.

### 3.2. **Variegation stability during *in vitro* propagation**

To evaluate the variegation stability and the influence of the medium and the number of multiplication cycles on it, the phenotype of the produced plants was observed and compared after three or six multiplication cycles, followed by one (YgVAR) or two (CaPP and PtJE) elongation cycles.

For *Yucca* 'Variegata', the variegation phenotype was clearly visible during all of the *in vitro* phases (Fig. 2A-D), whereas for *Cordyline* 'Pink Passion', the phenotype could only be determined after the elongation step (Fig. 2K, L). Since the *in vitro* plants of *Phormium* 'Jessie' do not express anthocyanins during the *in vitro* phase (Fig. 2E, F), their variegation was determined after acclimatization (Fig. 2G, H). Off-type plants corresponded to albino plants or to plants with excessively wide white edges for *Yucca* (Fig. 2D), non-variegated green leaves for *Phormium* (Fig. 2H), and non-variegated purple leaves (82 off-type plants obtained out of the 87 in total) or with an inverted variegation (5 off-type plants obtained out of the 87) for *Cordyline* (Fig. 2L).

The off-type rate is low for YgVAR, moderate for PtJE and high for CaPP (Fig. 3A, B and C, to the left, respectively). After six micropropagation cycles, the off-type rate was higher than after three cycles for all of the cultivars (9.77 and 11.10% vs. 1.01 and 2.33% for YgVAR (Fig. 5A left); 25 and 25.51% vs. 8.70 and 9.68% for PtJE (Fig. 3B left); and 60.98 and 62.50% vs. 45.45 and 31.25% for CaPP (Fig. 3C left) for BAP and NPA, respectively). No significant differences were found between the BAP and NPA combinations for the three cultivars (p-value > 0.05). For PtJE, on the control medium, the variegation was evaluated for the ten plants obtained after six cycles of multiplication. Among the ten plants obtained, three were off-type (30%), which is similar to the BAP and NPA results (p-value = 0.70, according to the Kruskal-Wallis test).

In order to determine whether the variegation losses are random or determined by the phenotype of the plants subcultured in each cycle, the phenotype of each daughter plant was compared to that of their

mother plant subcultured at the previous clonal cycle. Since the data were not significantly different between BAP and NPA, Table 2 summarizes the results of both media.

The mother plant phenotype is mainly transmitted to the daughter plants (Table 2). A true-to-type variegated plant produced 95.7%, 75.4% and 60.6% of true-to-type daughter plants for *Yucca, Phormium* and *Cordyline,* respectively. Similarly, an off-type mother plant produced 64.7%, 51.9% and 100% of off-type daughter plants for *Yucca, Phormium* and *Cordyline,* respectively, with no evolution of the off-type phenotype. Thus, for the three cultivars, there was no independence between the phenotypes of the mother plant and the daughter plant (p-value = 2.2e-16, 0.003694 and 3.344e-11 for YgVAR, PtJE and CaPP, respectively, with Chi² tests, Table 2). However, a return to the original true-to-type phenotype was observed from off-type plants for 35.3% and 48.1%, for *Yucca* 'Variegata', and *Phormium* 'Jessie', respectively. It was never observed for *Cordyline* 'Pink Passion'.

With the objective of determining the evolution of the ability of true-to-type plants to provide the same phenotype over several successive clonal cycles, an individualized monitoring of plants were performed, and made it possible to calculate the rate of off-type obtention from true-to-type shoots at each subculture (M1 to E1 or E2) (Fig. 3 right). For *Yucca* 'Variegata', the rate of off-type obtention remained low (under 2%) during the first four multiplication subcultures, regardless of the media. It significantly increased during M5 and persisted at around 5 and 7% during M6 and E1 (Fig. 3A right). For *Phormium* 'Jessie', the first multiplication subcultures produced only true-to-type plants during M2 with BAP and during M2 and M3 with NPA (Fig. 3B right). The rate of obtention of off-type plants per subculture then gradually increased to 30% or more on M6 and E1 (Fig. 3B right). For *Cordyline* 'Pink Passion', few true-to-type plants were observable after secondary shoot production at each subculture due to the low multiplication rate and the high mortality of explants. From M2 to E2, the percentage of off-type obtention per subculture varied from 10 to 100% with a high variability (Fig. 3C right). No impact of NPA on the rate of off-type obtention from true-to-type shoots was demonstrated for any of the three cultivars.

### 3.3. **Meristem origin during *in vitro* propagation**

To determine the origin of the new shoots, histological observations were made on samples during the multiplication cycles (Fig. S1). For each sample, the position of the shoot apical meristem was easily localized at the center between leaves (Fig. 4 A and J). Other meristematic areas were identified due to specific meristematic cell features: small isodiametric cells with a high nucleo-cytoplasmic ratio and a cytoplasm stained in blue due to its protein content with small or no detectable vacuoles (Fig 4 C).

For *Yucca* 'Variegata', these meristematic areas were all located along the stem at the underarm of the leaves, corresponding to the axillary bud meristem position (AxM, Fig. 4). On the control, the axillary buds were small (Fig. 4-A-J), without developed leaf primordia around the meristematic dome, with an approximate size of 100 µm (green arrows; Fig. 4-A and J, and Fig. S3). With PGR (BAP; Fig. 4-B-C), meristematic domes were larger (size greater than 200 µm), showing the active development of several leaf primordia (blue arrows; Fig. 4-B-C, and Fig. S3).

For *Phormium* 'Jessie', all meristematic areas (except the SAM) were also located along the stem at the underarm of the leaves for all conditions, which could again correspond to axillary bud meristems (AxM; Fig. 4). For the control, we can observe axillary meristematic areas (green arrows; Fig. 4D) with a size of 50 to 100 µm and with a few small leaf primordia green arrows; Fig. 4L. In conditions with PGR, a more active development of new shoots was visible (BAP; Fig. 4-E-F); axillary meristems presented a larger size, from100 to 150 µm, with more and larger leaf primordia (blue arrows; Fig. 4F). No differences were observed between BAP and NPA combinations and between SG0 (Fig. 4 D) and SG5 (Fig. 4E, and Fig. S4) shoots after the $6^{th}$ subculture.

For *Cordyline* 'Pink Passion', all meristematic areas (except the SAM) were located along the stem at the underarm of the leaves for samples on the control medium (AxM, green arrows; Fig. 4-G-H-I) with a size of approximately 50 µm and no leaf primordia (green arrows; Fig. S5). With PGR combinations, axillary buds were observable at the same location in the upper part of the stem (blue arrows; Fig. 4-H-I). However, in the lowest part of the stem, other meristematic areas were present, not necessarily at the underarm of the leaves. They consisted of a large meristematic dome (100 to 200 µm) and small leaf primordia (red arrows; Fig. 4K and M). Two types of buds, axillary bud meristems (AxM) in the upper part of the stem (blue arrows; Fig. 4-G-H-I) and adventitious bud meristems (AdM) in the lowest part, were observed (red arrows; Fig. 4H and K), and both developed secondary new shoots. Stems and

axillary buds presented a dense and continuous vascular system (green and blue arrows; Fig. 4-G-H-I), whereas in the lowest part of the shoot, the tissues induced by PGR combinations presented a less dense vascular system and did not necessarily continue until the appearance of the adventitious buds (red arrows; Fig. 4K and M).Observations performed on the entire width of the samples determined that only one axillary bud meristem was observed per leaf, for all combinations for *Yucca* and *Cordyline* (Fig. 4-A-B-C and Fig. 4-G-H-I, respectively), and for the control condition of *Phormium* (Fig. 4D), but that distinct areas of meristematic cells could be observed at the underarm of some leaves on media with PGR for *Phormium* (blue and red arrows; Fig. 4-E-F).

For all three cultivars, the differences between samples on the control medium and samples on PGR media could already be observed at 20 d and persisted up to the 6$^{th}$ cycle of SMM. Additional photographs of each condition are provided as supplementary data (Fig. S3, S4 and S5).

### 3.4. **Laser scanning confocal microscopy of leaf tissues**

To better characterize the nature of the variegation of these cultivars belonging to three different genera and to explain their degree of instability, laser scanning confocal microscopy was carried out on leaf tissues of the green wild types (YfWT, PtWT and CaWT respectively), true-to-type *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and *Cordyline australis* 'Pink Passion' (YgVAR TTT, PtJE TTT and CaPP TTT, respectively), and off-type plants derived from micropropagation of true-to-type plants: leaves with excessively wide white edges for YgVAR OT, uniformly green for PtJE OT, or purple for CaPP OT leaves (Fig. 5).

For *Yucca*, wild-type phenotype (YfWT), clearly revealed the presence of chloroplasts in the stomata guard cells (Fig. 5-A4-A5), whereas conversely, leaves of YgVAR (TTT and OT) did not reveal chlorophyll autofluorescence in the epidermis (Fig. 5-B4-B5 and C4-C5 respectively). Leaves of YfWT presented visible chloroplasts in all the layers of the mesophyll from the central rib (Fig. 5-A2) to the edge of the leaf (Fig. 5-A3). In YgVAR TTT, chloroplasts were visible in all the layers of mesophyll cells in the central part of the leaf blade (Fig. 5-B2), but with a mesophyll free of chloroplasts at the extreme of the leaf edge (Fig. 5-B3). Conversely, for leaves of YgVAR OT, one to two layers of

hypodermal cells were free of chloroplasts on the adaxial and abaxial side of the central rib (Fig. 5-C2), and the chloroplast-free leaf edges are much wider (Fig. 5-C3).

For *Phormium*, the three phenotypes (PtWT, PtJE TTT and PtJE OT) clearly revealed the presence of chloroplasts in the stomata guard cells with a high intensity of chlorophyll autofluorescence (Fig. 5-D4-D5, E4-E5, F4-F5, respectively). The three phenotypes all presented one to two layers of hypodermal cells free of chloroplasts on the adaxial side, from the central rib to the edge of the leaf (Fig. 5-D2, D3, E2, E3, F2 and F3). Leaves of PtWT and PtJE OT presented visible chloroplasts in all the other layers of the mesophyll, from the central rib (Fig. 5-D2 and F2, respectively) to the edge of the leaf (Fig. 5-D3 and F3, respectively). Conversely, for PtJE TTT, there was only one or two layers of cells with chloroplasts on the abaxial side of the central part (Fig. 5-E2), but the leaf edge presented cells with chloroplasts on the entire mesophyll, except for one or two layers of cells on the adaxial part of the leaf (Fig. 5-E3).

For *Cordyline*, the three phenotypes (CaWT, CaPP TTT and CaPP OT) clearly revealed the presence of chloroplasts in the stomata guard cells with a high intensity of chlorophyll autofluorescence (Fig. 5-G4-G5, H4-H5, I4-I5, respectively). Leaves of CaWT and CaPP OT presented visible chloroplasts in all the layers of the mesophyll from the central rib (Fig. 5-G2 and I2 respectively) to the edge of the leaf (Fig. 5-G3 and I3 respectively). On the other hand, for CaPP TTT leaves, one to two layers of hypodermal cells were free of chloroplasts on the adaxial and abaxial side of the central part of the leaf blade (Fig. 5-H2), and no edge cell contained any chloroplasts (Fig. 5-H3)Although anthocyanins fade rapidly, vacuoles with anthocyanins were observed in the first three-four hypodermal layers for all areas of the leaf of the CaPP TTT and OT (Fig. 5-H2-H5 and I2-I5 respectively). Anthocyanins fade too rapidly for *Phormium*, but were observed in the most internal mesophyll of the central part of PtJE TTT (Fig. 5-E2). No anthocyanin was visible in the leaves of *Yucca*, PtWT, PtJE OT or CaWT (Fig. 5-A2-A3, B2-B3, C2-C3, D2-D3, F2-F3 and G2-G3 respectively). Comparisons with photographs taken in white light and interpretative diagrams are provided as supplementary data (Fig. S6, S7 and S8).

# 4. DISCUSSION

## 4.1. *Yucca gloriosa*, *Phormium tenax* and *Cordyline australis* have different types of development

The aim of this study was to produce variegated and true-to-type plants. The most effective method for doing this consists of promoting the development of axillary buds (Chu, 1992; Vazquez, 2001; Zayova et al., 2010; Phillips and Garda, 2019). Nevertheless, some regeneration can also arise from adventitious meristems; this dual origin is referred to as "mixed cultures" (George and Debergh, 2008). Since the origin of newly formed axes (axillary buds, adventitious organogenesis) can have an impact on variegation stability, it was determined on the basis of detailed histological observations.

For the three cultivars, *Yucca gloriosa*, *Phormium tenax* and *Cordyline australis*, histological observations revealed that each leaf presents a single and unique axillary bud at its underarm, as is the case for many other monocotyledons (Fisher, 1978; De Klerk, 2012). The three species revealed different growth behaviors *in vitro* with or without PGR (Fig. 6).

Without PGR, the plants developed similarly to plants *in vivo*. For *Yucca gloriosa* and *Cordyline australis,* the axillary buds are subjected to apical dominance (Fig. 6), which is naturally released upon formation of the flowering stem, and whose development leads to the budburst of the axillary buds and secondary branching (Tomlinson and Fisher, 1971). For *Phormium tenax*, this dominance is lesser and presents a gradient of intensity that allows the budburst of the most distant axillary meristems, with a basitonic development (Fig. 6), similar to its development *in vivo* with many shoots (Pal Puri et al., 1966).

BAP is a synthetic cytokinin that stimulates cellular division and control of morphogenesis, making it possible to inhibit apical dominance, to release lateral buds from dormancy and to stimulate the development of new adventitious meristems (George and Debergh, 2008). In the presence of this PGR, *Yucca gloriosa* presented a propagation exclusively linked to the development of axillary bud meristems in (Fig. 6). *Phormium tenax* had a majority of shoots arising from axillary bud meristems, with several

non-axillary shoots arising from distinct cell groups with a meristematic appearance and located close to the axillary meristem bud (Fig. 6), referred to as "semi-axillary" and generally classified as adventitious regeneration (De Klerk, 2012). Finally, for *Cordyline australis*, exposure to BAP led to the propagation of axillary buds in the upper part of the plant, but also stimulated the formation and the development of new adventitious bud meristems in the basal part of the plant by direct organogenesis (George and Debergh, 2008; Fig. 6). Consequently, *Phormium tenax* and *Cordyline australis* presented both axillary and adventitious shoots, referred to as "mixed cultures" by George and Debergh (2008).
A concentration of 4.44 µM of BAP was sufficient to allow the stimulation of axillary and adventitious bud meristems, even in different proportions, in *Phormium tenax* and *Cordyline australis*. However, for *Yucca gloriosa*, despite a 15x higher concentration (66.59 µM), only axillary bud meristems developed.
NPA is an auxin transport inhibitor that reduces apical dominance to promote axillary outgrowth (Teale and Palme, 2018). Although the addition of 1 µM of NPA did not seem to have had an effect on the behavior of the three species, no differences were observed with medium supplemented with NPA during histological observations or on the conformity of the plants obtained.

### 4.2. *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and *Cordyline australis* 'Pink Passion' are periclinal chimeras with different contributions of layers to leaf development

The three variegated cultivars present bicolored leaves with a difference between the center of the limb and the leaf edges. These variegated models (cell-lineage type) are generally associated with the expression of genes differentiated according to their location on the leaf or their epigenetic instability (Marcotrigiano, 1997; Nabeshima et al., 2017), but more specifically with periclinal chimeras, i.e., plants where an entire layer of the shoot apical meristem is genetically distinct from the others (Stewart and Dermen, 1979; Marcotrigiano, 1997; Frank and Chitwood, 2016). The three layers (L1-L2-L3) of the meristem are maintained during plant development and contribute to the formation of the different organ tissues, where the L1 layer normally contributes to the epidermis, the L2 layer to one or two layers

484 of subepidermal cells and the leaf edge mesophyll, and the L3 layer to the inner leaf blade mesophyll
485 (Fig. 7). In leaves, when a layer is genetically different in terms of anthocyanin synthesis or chloroplast
486 biogenesis, a phenomenon of variegation can occur (Stewart and Dermen, 1979; Marcotrigiano, 1997;
487 Frank and Chitwood, 2016). To confirm the nature of the variegation and explain the potential instability
488 during propagation, a characterization of the different leaf tissues was carried out.
489 The histological observation of different phenotypes with green leaves (YfWT, PtWT and CaWT),
490 variegated (YgVAR TTT, PtJE TTT and CaPP TTT) with an over-expression of the variegation
491 (YgVAR OT), or with the loss of variegation (PtJE OT and CaPP OT), enabled us to highlight exactly
492 three layers of uniform cells on these species. On the basis of these results, it could be concluded that
493 the three variegated cultivars, *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and *Cordyline*
494 *australis* 'Pink Passion', are periclinal chimeras with three distinct meristematic layers, L1-L2-L3, with
495 different contributions of these meristematic layers to leaf development, depending on the species.
496 Results reveal that *Yucca gloriosa* 'Variegata' is a periclinal chimera with a chloroplast-deficient layer
497 and a W-G-G chimeric structure (L1-L2-L3, G and W, corresponding to layers with a uniform
498 development (green) or a deficiency in chloroplasts (white), respectively), where the L1 layer
499 contributes to the leaf border mesophyll (Fig. 8), as in *Chlorophytum* and *Dracaena* (Stewart and
500 Dermen, 1979). The OT phenotype with over-expression of the variegation is then the result of a
501 periclinal chimera in W-W-G, with chloroplasts maintained only in the innermost L3 layer (Fig. 8),
502 which explains the wider edges and the light green phenotype of the leaves. Observations also lead to
503 the conclusion that *Phormium tenax* 'Jessie' is a periclinal chimera with a chloroplast-deficient layer and
504 a G-G-W chimeric structure, where the L1 layer contributes to the epidermis, the L2 layer to one or two
505 layers of subepidermal cells and to the mesophyll of the leaf edge, and the L3 layer deficient in
506 chloroplasts but enriched in anthocyanins, contributes to the internal mesophyll of the leaf blade (Fig.
507 8). However, the presence of a hypodermic thickness without chloroplasts, characteristic of the genus
508 *Phormium* (Pal Puri, 1966), "masks" the contribution of the L2 layer on the adaxial surface (Fig. 8). The
509 phenotypes studied do not make it possible to determine if the L1 layer is involved only in the epidermis
510 or in the mesophyll of the leaf edges as well. The non-variegated OT phenotype is then the result of
511 three G-G-G layers, and therefore to the loss of a chimeric structure, leading to a uniformly green

phenotype (Fig. 8). The cultivar ‘Pink Passion’ of *Cordyline australis* appears to be a periclinal chimera with a layer deficient in chloroplasts and a chimeric structure in G-W-G. The L1 layer contributes only to the epidermis, and the L2 layerto two-three layers of subepidermal cells and to the mesophyll of the leaf edges (Fig. 8). The non-variegated OT phenotype is therefore the result of three G-G-G layers and, consequently, the loss of a chimeric structure. The phenotype with reversed variegation is the result of a G-G-W chimera with edges derived from an L2 layer with chloroplasts and an L3 layer deficient in chloroplasts that contributes to the most internal mesophyll at the center of the leaf blade (Fig. 8).

These results have thus made it possible to highlight new knowledge about the leaf development of *Yucca gloriosa* and *Cordyline australis* species, with different contribution patterns of the L1 layer to leaf development. While *Cordyline*, an *Asparagaceae*, grows like the majority of monocot and dicotyledonous plants, *Yucca gloriosa* behaves like some *Dracaena* and *Chlorophytum*, other Asparagales, with a contribution of L1 to the border mesophyll (Stewart and Dermen, 1979), allowing the creation of new variegation patterns.

Likewise, although the different phenotypes of *Phormium* did not allow to study the contribution of L1, the histological study of its leaves made it possible to explain the highly contrasted and economically valued variegation of *Phormium tenax* 'Jessie': (1) a double mutation affecting a deficiency of chloroplasts and anthocyanin synthesis that generates a highly-colored pink meristematic layer; and (2) the presence of a hypodermic thickness without chloroplasts, characteristic of the genus *Phormium* (Pal Puri, 1966), which reveals the phenotype of the more internal L3 layer by “masking” the L2 layer, and therefore allows a very strong contrast between the highly colored pink leaf blade and the green edges.

### 4.3. Variegation stability is influenced by the origin of new shoots and the cause of variegation

Periclinal chimeras remain stable when propagation does not provoke the reorganization of cell layers (Marcotrigiano, 1997; Frank and Chitwood, 2016). Adventitious meristem formations generally arise from a single cell in many plants (Boertjes et al., 1968; Yang et al., 2017), often with only the

participation of the L1 epidermal layer (Boertjes and Van Harten, 1985; Peary et al., 1988; Yang et al., 2017). Nevertheless, histological analyses revealed that all of the cell layers can be at the origin of an adventitious meristem, with the possibility of a multicellular origin, sometimes leading, even in small proportions, to variegated plants arising from adventitious regeneration (Marcotrigiano, 1986; Nabeshima et al., 2017). The consequence is that numerous studies have shown that propagation of periclinal chimeras by axillary bud meristems maintained a chimeric structure, whereas adventitious meristems led to the reorganization of cell layers and to very low rates of true-to-type plants (Papachatzi et al., 1981; Pierik et al., 1983; Lineberger and Wanstreet, 1983; McPheeters and Skirvin, 1983; Lineberger and Druckenbrod, 1985; Marcotrigiano, 1986; Pogany and Lineberger, 1990; Marcotrigiano et al., 1997; Amory and Gill, 1999).

Since all three of the cultivars are periclinal chimeras, variegation stability could be therefore directly correlated with the *in vitro* behavior of the cultivars, and with an increase in non-conformity associated with the varying degree of propensity of the cultivar to propagate via adventitious meristems. A high level of stability is obtained in *Yucca gloriosa* 'Variegata' (W-G-G), whose propagation depends on the axillary bud meristems (Fig. 6). The off-type phenotype with overexpression of the variegation (W-W-G) could be the consequence of the "replacement" of L2 by L1 (Stewart and Dermen, 1970) during the development of the meristem, which remains a point phenomenon (Marcotrigiano, 1997). Moderate stability is obtained in the periclinal chimera *Phormium tenax* 'Jessie' (G-G-W), whose propagation depends on a majority of axillary bud meristems and several adventitious meristems (Fig. 6) where off-type plants correspond to G-G-G, probably arising from a single cell of L1 or from a multicellular origin between L1 and L2.Finally, low stability is obtained in *Cordyline australis* 'Pink Passion' (G-W-G) with its development in "mixed culture", depending on both axillary and adventitious bud meristems (Fig. 6). We can therefore assume that true-to-type plants were mainly obtained by axillary bud meristems, whereas off-type plants were, in part, obtained by adventitious bud meristems. Consequently, it can be hypothesized that the appearance of purple and non-variegated off-type plants (82 plants out of the 87 off-type plants obtained) corresponds to G-G-G plants (Fig. 8), probably arising from a single cell in the L1 epidermal layer in G, whereas the few off-type plants with a reversed variegation (5 plants

566 out of the 87) correspond to G-G-W chimeras (Fig. 8), probably arising from a multicellular origin
567 between the L1 and L2 layers, with a greater contribution of the L1 layer.

568 ### 4.4. Necessity of regularly sorting off-type plants at each subculture

569

570 Plant-to-plant monitoring of the conformity of plants obtained at each clonal cycle made it possible to
571 highlight an increasing frequency of the number of off-type plants at each new subculture for *Yucca* and
572 *Phormium*, and the extremely variable and high rates of loss of conformity at each of the cycles for
573 *Cordyline*. It is however interesting to note that these rates do not seem to increase for *Cordyline*, which
574 is highly variable from the first to the last clonal cycles, nor for *Yucca* beyond the 5th subculture. It is
575 all the more important since this study has shown that an off-type plant mainly or exclusively gives rise
576 to off-type plants at the following cycle, thus emphasizing the interest in the possibility of eliminating
577 them during the growth cycle.
578 This selection would nevertheless be difficult for *Phormium,* which expresses few anthocyanins in the
579 *in vitro* phase, and for *Cordyline,* whose leaves do not unfold before the elongation phase, making
580 conformity observations difficult (Fig. 2F, J). The development of more complex multiplication
581 protocols with intermediary elongation phases, for example, or the adaptation of culture media to allow
582 elongation and/or leaf unfolding should be studied in greater depth in order to improve this selection.

583

584 ### 4.5. Multiplication of *Yucca gloriosa* 'Variegata', *Phormium tenax* 'Jessie' and
585 *Cordyline australis* 'Pink Passion'

586

587 While multiplication rates on the order of 3 to 4 for cycle time of 3 weeks have been identified in *Yucca*
588 *valida* (Arce-Montoya et al., 2006), the rates obtained in this study are on the order of 2.5 to 3, which is
589 slightly lower, but still allows a faster production than the rates of 6 to 8 obtained for various *Yucca*
590 species by lengthening the cycle time to 5 to 12 weeks (Pierik and Steegmans, 1983; Bentz et al., 1988;
591 Atta-Alla and Van Staden, 1997).

592 High levels of multiplication have been reported in the literature for *Cordyline*, with, e.g., 6 for
593 *Cordyline sp*. (Chinnu et al., 2012), 14 for *C. fruticosa* (Dewir et al., 2015) and even 60 buds on five
594 weeks for *C. terminalis* (Ray et al., 2006). In this study, a very low level, between 1.2 and 1.7, was
595 obtained for *Cordyline australis*. This difference in multiplication levels should be due to: (1) the quality
596 of our plant material, i.e., differences that could be explained by the physiological or sanitary status of
597 the mother plants; (2) the genotype, even within the same genus, many differences in vigor and
598 multiplication can be observed between genotypes as shown for *Vaccinium* (Fan et al., 2017), *Musa*
599 (Selvakumar et Parasurama, 2020), or *Prunus* (Khafri et al., 2020); (3) the variegation phenotype, i.e.,
600 it is recognized that variegation has a negative impact on vigor and plant development due to a reduction
601 in photosynthetic capacity in chloroplast-deficient tissues (Sheue et al., 2012); and (4) *in vitro* conditions
602 since the cytokinin-type (BAP) appeared to induce the formation of meristems, but inhibit the elongation
603 of the stem and the leaf system. Indeed, too high levels of cytokinin cause many small shoots, which
604 typically fail to elongate, and may also cause an unusual shape of the leaves of some species (Gaba,
605 2004; van Staden et al., 2008; Martini and Papafotiou, 2013; Geng et al., 2016). In this study, elongation
606 of *Cordyline* mainly developed with a last subculture on a medium devoid of cytokinin. The addition of
607 Gibberellin (GA3) could be considered to stimulate elongation during the multiplication phase and, at
608 the same time, promote the sorting of off-type shoots; previous studies on *Cordyline* have shown an
609 optimal concentration of 5mg/L for elongation (Chinnu et al., 2012). *Phormium* showed low
610 multiplication rates on the order of 1.4 to 1.7, but no comparative tissue culture studies were found,
611 probably explained by the relative ease of *in vivo* multiplication of the genus *Phormium*.
612 Although naphthylphthalamic acid (NPA) is one of the most popular auxin transport inhibitors used to
613 reduce apical dominance and increase multiplication rates, many questions remain, e.g., about its
614 binding sites to specific proteins, the proximity of its binding to the transporter, and even if it can be
615 considered functionally equivalent to endogenous inhibitors (Teale and Palme, 2018). Studied as a
616 treatment at high concentration on cultures, it nevertheless enhanced axillary outgrowth on pseudobulbs
617 of *Cremastra appendiculata* (Lv et al., 2018) and in the micropropagation of *Citrus sp.* (Hu et al., 2017).
618 In the tissue culture of *Alstroemerias*, a monocotyledon, axillary outgrowth was gradually increased
619 with a treatment of between 0 and 10 µM, but decreased after 10 µM (Pumisutapon, 2012). In this study,

620 the addition of 1 µM of NPA to BAP increased the mean multiplication rate in *Yucca*, but not in
621 *Phormium* and *Cordyline*. This could be due to the difference in sensitivity to NPA depending on the
622 genus, as well as to the difference in BAP concentration. No sign of NPA toxicity was observed at a
623 concentration of 1 µM.
624 Detailed monitoring of the material made it possible to determine the multiplication rate of each
625 generation of plants obtained over the six multiplication cycles and to highlight the differences in
626 behavior and *in vitro* development in these three botanical genera. Unlike *Yucca* where all the new plants
627 also participate in rapid multiplication, for *Phormium* and *Cordyline*, it was the initial explants (SG0
628 mother plants) that had the highest multiplication rates. Several hypotheses can be proposed: (1) the
629 internal vigor of the initial explants; (2) cycles of 4 weeks that were too short, with too many successive
630 subcultures before the elongation of new shoots; (3) better development of the foliar system and better
631 growth and multiplication of plants when a phase without PGR is applied (in this case, an introduction
632 step for the SG0); and (4) in the case of *Cordyline*, multiplication present but not visible before
633 elongation (buds in the basal part). Unlike *Yucca* and *Phormium*, *Cordyline* exhibits *in vitro*
634 multiplication behavior in the form of a cluster of meristems, subsequently requiring an essential
635 elongation phase.
636 Further investigation of increased NPA concentrations and potential new regulators that promote the
637 development of axillary meristems could both increase the multiplication rate and promote propagation
638 via axillary buds favorable to maintaining conformity. Only the improvement of these two objectives
639 would make the production of these variegated cultivars of *Cordyline* and *Phormium* industrially viable.
640 Experiments carried out with TIBA (2,3,5-triiodobenzoic acid, another inhibitor of auxin transport), D2
641 (an inhibitor of the oxidative cleavage of carotenoids), fluridone (a carotenoid biosynthesis inhibitor that
642 reduces the production of strigolactone), or ethephon (which decomposes to the PGR ethylene in
643 aqueous solutions) have shown a strong effect on axillary bud outgrowth on several monocotyledons in
644 tissue culture (Pumisutapon, 2012; Keshavarzi, 2017; Shahin et al., 2018).
645 Moreover, subsequent LAP research into the extension of cycle length for *Phormium* has made it
646 possible to increase the multiplication rate, revealing the impact of the length of each cycle and allowing
647 us to consider the production of this cultivar (pers. communication of LAP).

### 4.6. Industry point of view for a necessary protocol optimization for *Phormium tenax* and *Cordyline australis*

For the purpose of industrial production, a minimum multiplication rate of 2, which remains constant from one cycle to the next, is necessary. These multiplication rates are satisfactory for *Yucca*, but they remain much too low for *Phormium* and *Cordyline*. Because of their impact on the number of plants that need to be transplanted at each cycle to obtain a specific number of plants, they therefore have an influence on labor costs, which constitute the major part of the expenses involved in *in vitro* production systems. In contrast, since the costs generated by growth chambers are the lowest (particularly in Western countries), an extension of the length of growth cycles would lead to little additional cost compared to the gains obtained in labor due to the increase in the multiplication rates in the case of *Phormium tenax*.

Moreover, even if laboratories can potentially accept losses of 5-10% in order to remain economically viable, cultivars of *Phormium* and *Cordyline* have also revealed excessively high off-type plant rates. Since the loss of plant conformity is directly correlated with the capacity of plants to propagate via adventitious bud meristems, it appears to be necessary to encourage the development of axillary meristems, on the one hand, to decrease the non-conformity rate and, on the other, to increase the multiplication rate. The increase of NPA concentrations and potential new regulators (TIBA, fluridone) are also avenues that remain to be explored. In an industrial context, the additional cost of the use of these molecules, even in high concentrations of up to 10 µM, would be on the order of €0.001 to €0.006/plant. If they make it possible to reach the multiplication rate of 2, this is an extremely negligible cost linked to gains in productivity in terms of labor (calculated according to the catalogue prices of Sigma-Aldrich, Lyon, France, and Appolo, Bredbury, England).

672 # 5. Conclusion

673

674 The three cultivars studied here for their highly attractive and desirable variegated foliage turned out to
675 be three chimeras, a structure known to be particularly difficult to consistently propagate in *in vitro*
676 culture, especially via adventitious meristems. The histological study carried out on three different
677 genera contributes to identifying the major differences in the development and behavior of the *in vitro*
678 multiplication of monocotyledons. The varying degree of the propensity to propagate via adventitious
679 meristems can therefore be correlated with the variegation stability levels of chimeric plants. With the
680 exception of *Yucca*, the multiplication and conformity rates do not allow us to envision production at
681 the industrial level, but the high correlation between the phenotype of daughter plants and mother plants
682 at each subculture implies the necessity of sorting off-type plants as early as possible. Nevertheless, the
683 absence of elongation of new shoots prevents us from observing variegation before the end of the
684 multiplication process. It would therefore be beneficial to improve elongation by extending growth
685 cycles or by the addition of gibberellins. In the same way, in order to promote the development of
686 axillary bud meristems in the aim of increasing (1) the proportion of the number of true-to-type plants
687 obtained, and (2) the multiplication rate, it would be interesting to explore the use of auxin transport
688 inhibitors (NPA and TIBA) and inhibitors of strigolactone (D2 and fluridone) biosynthesis in the *in vitro*
689 multiplication process of these cultivars. This study also made it possible to highlight new knowledge
690 about leaf development of *Yucca gloriosa* and *Cordyline australis* species, with different contribution
691 patterns of the meristem layers in leaf development, and to explain the causes of the very contrasting
692 variegation of cultivars of the genus *Phormium* and their great economic interest.

693

## Author contributions

Alexandre Rouinsard: Investigation, Formal analysis, Writing - Original Draft; Latifa Hamama : Writing – Review & Editing, Supervision; Laurence Hibrand-Saint Oyant: Writing - Review & Editing, Supervision; Agnès Grapin: Conceptualization, Writing - Review & Editing, Supervision, Project administration.

## Declaration of interest

The authors declare that they have no conflict of interest.


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## Supplementary data

Supplementary data associated with this article can be found, …

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Figure 1: Phenotypes of *Yucca gloriosa* 'Variegata' (YgVAR) with green leaves and thin white edges (A), *Phormium tenax* 'Jessie' (PtJE) with green leaves and a pink stripe in the center (B), and *Cordyline australis* 'Pink Passion' (CaPP) with purple leaves and pink edges (C). Scale bar = 1 cm.

Figure 2: Micropropagated shoots of *Yucca gloriosa* 'Variegata' (A, B, C, D), *Phormium tenax* 'Jessie' (E, F, G, H), and *Cordyline australis* 'Pink Passion' (I, J, K, L). Photographs represent *in vitro* plants during subculture M6 on media supplemented with PGR with explants SG0 (A, E, I) and SG5 (B, F, J), true-to-type (C, G, K) and off-type plants (D, H, L), respectively, on multiplication medium with PGR (D), on rooting medium after elongation (C, K, L), and after acclimatization (G, H) for *Phormium*. Blue arrows indicate new buds,black arrows new shoots, and the red arrow a new off-type shoot. Scale bar = 1 cm.

Figure 3: Total rate of off-type plants (%) produced after three or six cycles (left), and evolution of the rate of off-type plants obtained from true-to-type shoot tips at each cycle (right) on BAP or NAP multiplication medium for (A) *Yucca* 'Variegata' (YgVAR), (B) *Phormium* 'Jessie' (PtJE), and (C) *Cordyline* 'Pink Passion' (CaPP). Multiplication media (M1 to M6): BAP: medium supplemented with BAP (66.59 µM for YgVAR; 4.44 µM for PtJE and CaPP); and NPA medium: medium supplemented with BAP (at the same concentrations), and 1 µM of NPA. Shoot elongation medium (E1 and E2): PGR-free medium for all plants. Off-type plants correspond to: albino plants or those with excessively wide white edges for YgVAR, non-variegated green leaves for PtJE, and non-variegated purple leaves or with an inverted variegation for CaPP. Phenotypic observation was performed at the end of the rooting step for *Yucca* and *Cordyline*, and during the acclimatization step for *Phormium*. For the rate of off-type plants obtained from true-to-type shoot tips (right), only plants obtained from true-to-type shoot tips were considered (plants descended from off-type shoot tips or from shoot tips for which the phenotype could not be observed, e.g., the shoot tip died during the process, were excluded). Bimodal phenotype data (i.e. 0 for true-to-type and 1 for off-type phenotype respectively) were fitted in logistic models with taking as reference the lowest result: "***", "**", "*" and "." represent p-value < 0.001, 0.01, 0.05 and 0.1 respectively. Bars represent the standard error of the mean.

Figure 4: Histological observations of *in vitro* explants of *Yucca gloriosa* 'Variegata' (A, B, C, J), *Phormium tenax* 'Jessie' (D, E, F, L), and *Cordyline australis* 'Pink Passion' (G, H, I, K, M, N). Photographs represent SG0 shoot tips on Control medium during cycle M6 (A, D, G) and at 20 d of clonal cycle M1 (J, L), and shoot tips on medium with PGR (B, C, E, F, H, I, K, M, N) with SG0 shoot tips on BAP mediumat 20 d (C, F) or during clonal cycle M6 (B, H, I, K), and SG5 shoot tips on NPA (E) or BAP medium (M, N). Black arrows indicate the shoot apical meristem (SAM), blue arrows indicate the axillary bud meristems (AxM), green arrows indicate axillary bud meristems (AxM) repressed by the SAM, and red arrows indicate adventitious bud meristems (AdM). Scale bar = 1 mm (A, B, E, G, H, M), 500 µm (D, J), 200 µm (N) or 100 µm (C, F, I, K, L).

Figure 5: Leaf analysis of fresh leaves of (A) *Yucca gloriosa* non-variegated wild-type (YfWT), (B) True-to-type *Yucca gloriosa* 'Variegata' (YgVAR TTT), (C) Off-type *Yucca gloriosa* 'Variegata' (YgVAR OT), (D) *Phormium tenax* non-variegated wild-type (PtWT), (E) True-to-type *Phormium tenax* 'Jessie' (PtJE TTT), (F) Off-type *Phormium tenax* 'Jessie' (PtJE OT), (G) *Cordyline australis* non-variegated wild-type (CaWT), (H) True-to-type *Cordyline australis* 'Pink Passion' (CaPP TTT) and (I) Off-type *Cordyline australis* 'Pink Passion' (CaPP OT). Photographs represent a fresh leaf (A1, B1, C1,…, I1), and leaf transections observed with a confocal laser scanning microscope with excitations provided by a laser at 405 nm (blue), 561 nm (yellow) and 638 nm (red) to observe the lignin and cuticle, anthocyanins, and chlorophyll autofluorescence respectively(A2-I2, A3-I3, A4-I4, A5-I5). Photographs represent pseudo-color images with excitations provided by laser at the central rib (A2-I2), the edge of the leaves (A3-I3), and a random region with stoma (A4-I4). The same photographs of A4-I4 are represented without pseudo-color image to reveal the autofluorescences on a black background (A5-I5). Red and white arrows represent stomata that do or do not contain chloroplasts, respectively. The scale bar = 2mm (A1-I1), 100 µm (A2-I2 and A3-I3) or 25 µm (A4-I4 and A5-I5).

Figure 6: *In vitro* behavior of different species depending on the culture medium. Shoot apical meristems (SAM) are represented in black, axillary bud meristems (AxM) repressed by the SAM or that develop new shoots are represented in green and blue, respectively, and adventitious bud meristems (AdM) are represented in red. The arrows indicate bud meristems that develop new shoots.

Figure 7: Schematic representation (A) of the meristem structure; (B) of the meristem contribution to leaf development: the L1 layer contributes to the epidermis, the L2 layer to the palissadic parenchyma and the mesophyll of the leaf edge, and the L3 layer to the internal mesophyll (from Stewart and Dermen, 1979; McHale and Marcotrigiano, 1998).

Figure 8: Candidate pattern for the meristem contribution to leaf development and chimeric structure of the different phenotypes obtained by micropropagation. Yg: *Yucca gloriosa*; YgVAR: *Yucca gloriosa* 'Variegata'; Pt: *Phormium tenax*; PtJE: *Phormium tenax* 'Jessie'; Ca: *Cordyline australis*; CaPP: *Cordyline australis* 'Pink Passion'; WT: Wild-Type; TTT: True-to-type; OT: Off-Type; G: Green layer of the meristem with a good development of chloroplasts; W: White layer of the meristem with a deficient development of chloroplasts. For each chimeric structure, the letter order represents the layers L1, L2 and L3, respectively.

Table 1: Evolution of the multiplication rate according to the PGR combination, cycle and Shoot Generation for (A) *Yucca* 'Variegata' (YgVAR), (B) *Phormium* 'Jessie' (PtJE) and (C) *Cordyline* 'Pink Passion' (CaPP). The multiplication rate corresponds to the number of individualized shoots obtained per each cultivated shoot tip (dead plants are not subcultured and are therefore not considered). BAP, NPA and Control indicate shoots cultivated during the multiplication cycles (M1 to M6) on a medium supplemented with BAP (66.59 µM for YgVAR; 4.44 µM for PtJE and CaPP), with BAP (at the same concentrations) and 1 µM of NPA, or on PGR-free medium, respectively. Shoot elongation cycles (E1 for YgVAR, E1 and E2 for PtJE and CaPP) were on PGR-free medium for all plants. Shoot Generat.: Shoot Generation; No. plants: number of shoot tips cultivated at the beginning of each cycle; No. deads: number of shoot tips dead during the clonal cycle and not subcultured for subsequent cycle; Tot. M5: Total of the different multiplications during clonal cycle M5, for all generations; se: Standard-Error. Means of multiplication rate that are not connected by the same letter are significantly different at 0.05 probability level by Tukey's HSD test.

[a] A first Tukey's HSD test performed on the different generation modalities according to the medium and the clonal cycles, illustrated by the letters "a" to "i"

[b] A second Tukey's HSD test performed on the different clonal cycles according to the medium, illustrated by the letters "A" to "F"

[c] A third Tukey's HSD test performed on the different medium BAP, NPA and Control media throughout the entire duration of the experiment, illustrated by the letters "A" to "C"

Table 2: Phenotype of micropropagated plants obtained according to the parental phenotype at the end of the micropropagation process, for all medium compositions.

[a] Green leaves with thin white edges, purple leaves with pink edges, and green leaves with a pink stripe generally in the center, for *Yucca* 'Variegata', *Cordyline* 'Pink Passion' and *Phormium* 'Jessie', respectively.

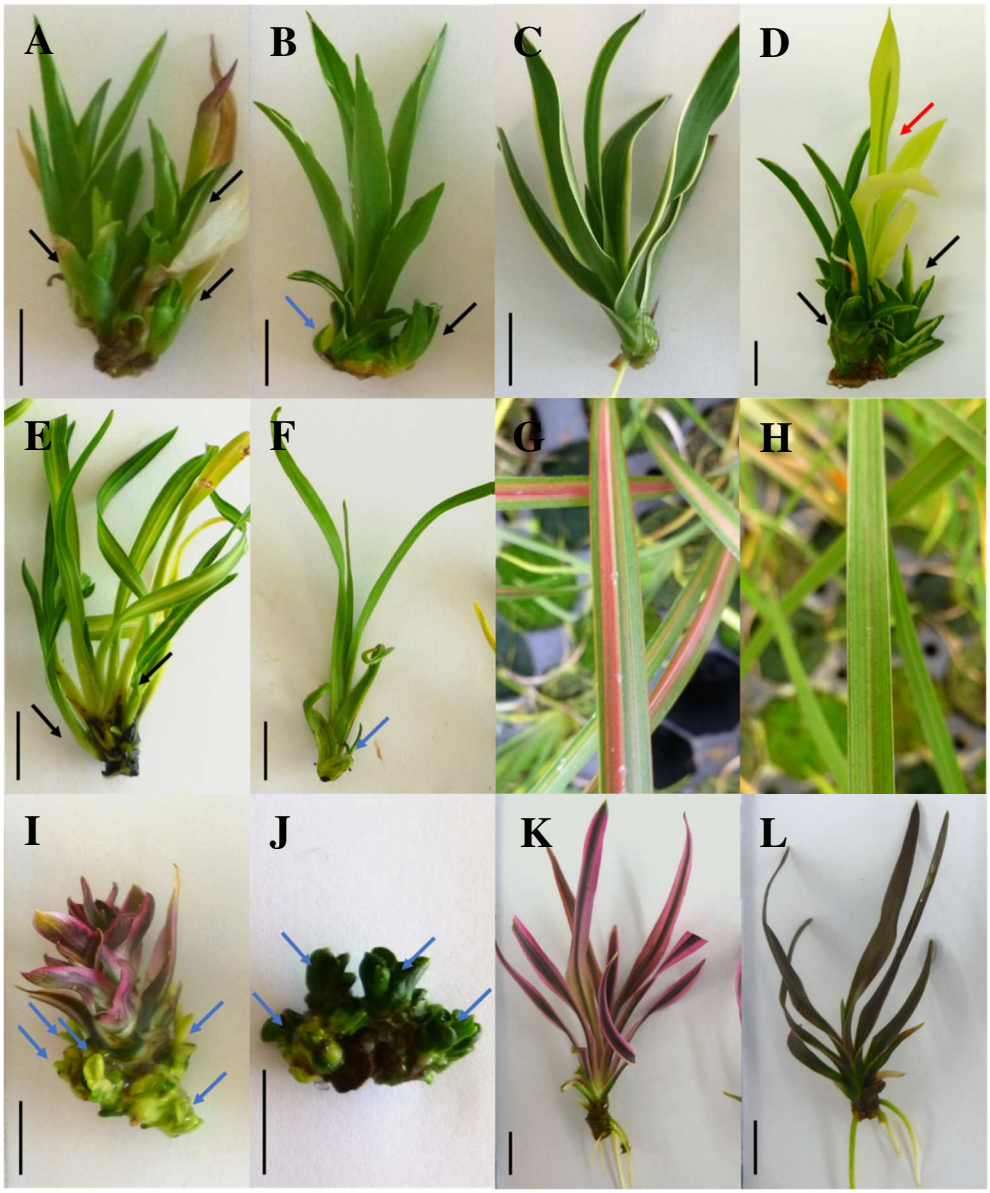
[b] Albino plants or those with excessively wide white edges, non-variegated purple leaves or with an inverted variegation and non-variegated green leaves, for *Yucca* 'Variegata', *Cordyline* 'Pink Passion' and P*hormium* 'Jessie', respectively.

[c] The phenotype of micropropagated and parent explants was measured at the end of the micropropagation process, during the rooting step.

[d] The phenotype of micropropagated and parent explants was measured during the acclimatization step because explants do not present any anthocyanin during the *in vitro* process.

Fig. 1

**Fig. 2**




934

**Fig. 3**

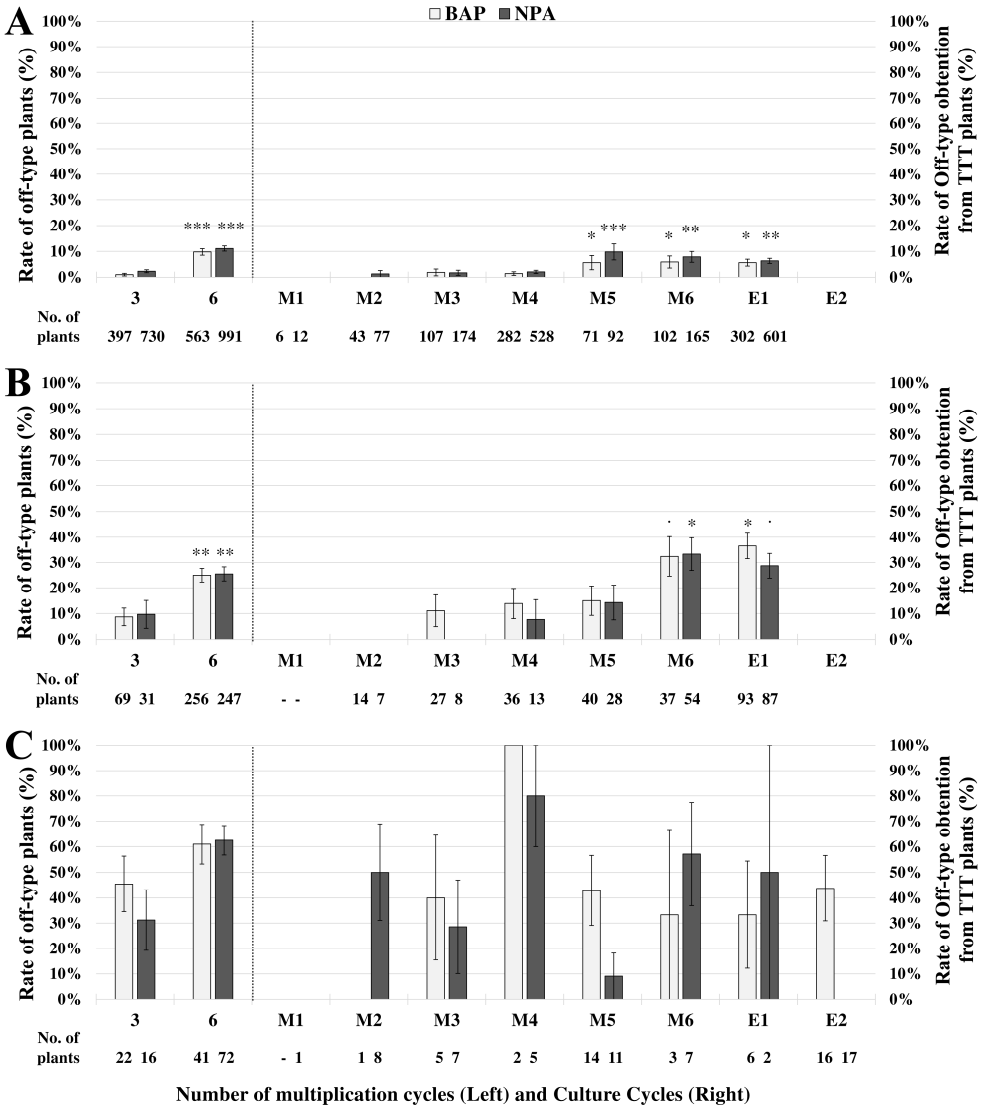

A
B
C
BAP
NPA
Rate of off-type plants (%)
Rate of Off-type obtention from TTT plants (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
3
6
M1
M2
M3
M4
M5
M6
E1
E2
No. of plants
397 730
563 991
6 12
43 77
107 174
282 528
71 92
102 165
302 601
69 31
256 247
- -
14 7
27 8
36 13
40 28
37 54
93 87
22 16
41 72
- 1
1 8
5 7
2 5
14 11
3 7
6 2
16 17
Number of multiplication cycles (Left) and Culture Cycles (Right)

Fig. 4

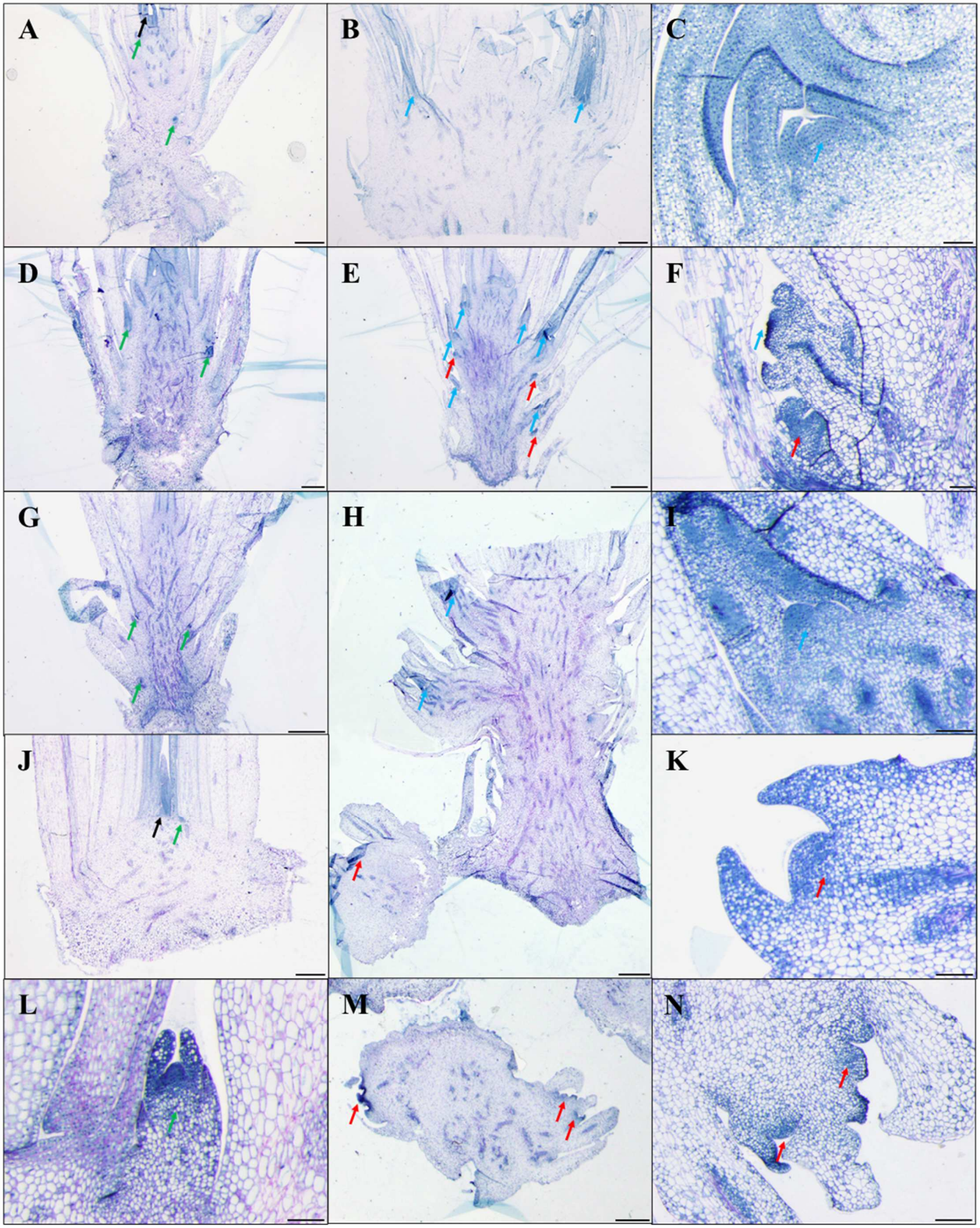


Fig. 5

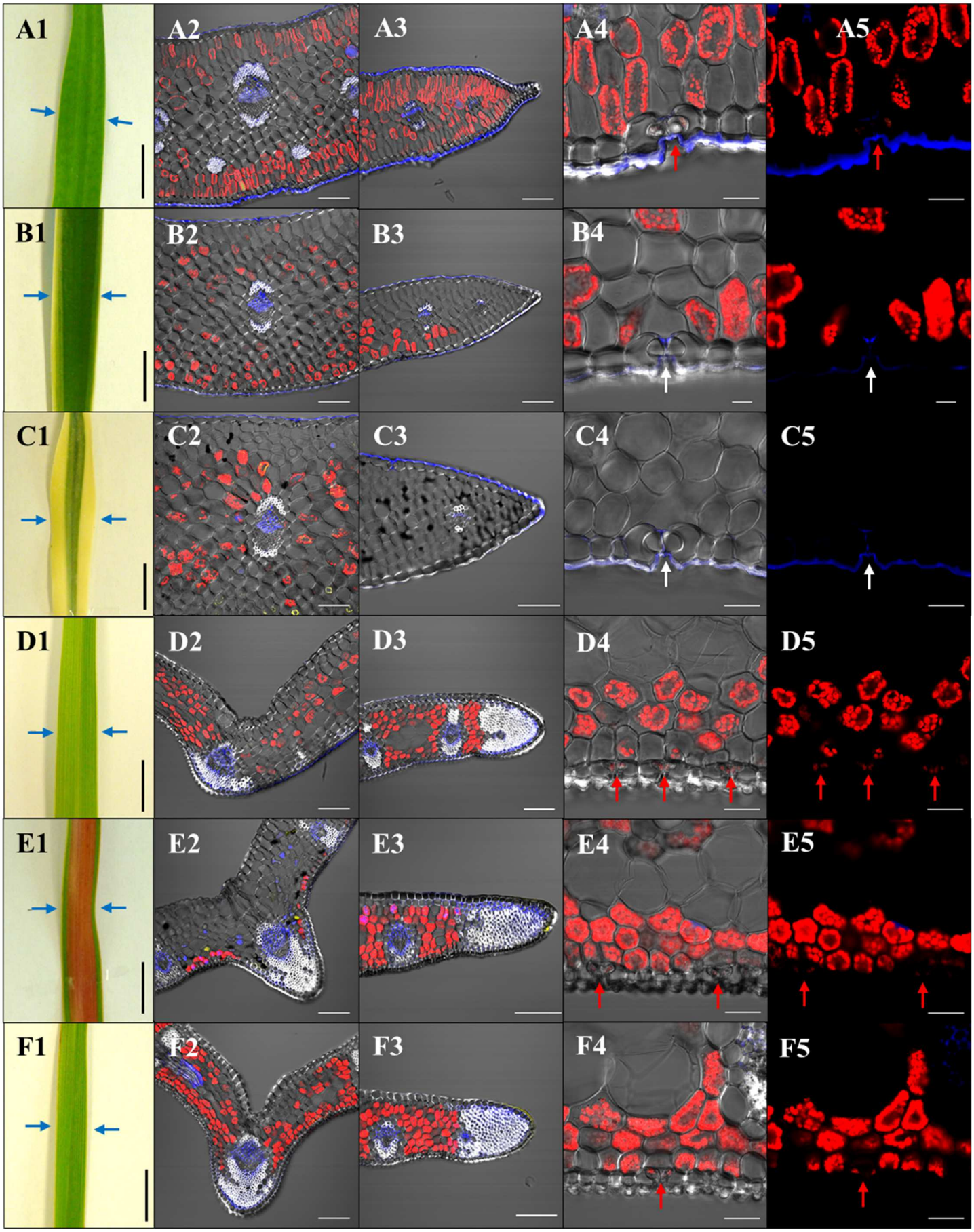

A1
A2
A3
A4
A5
B1
B2
B3
B4
C1
C2
C3
C4
C5
D1
D2
D3
D4
D5
E1
E2
E3
E4
E5
F1
F2
F3
F4
F5

**Fig. 5 (continued)**

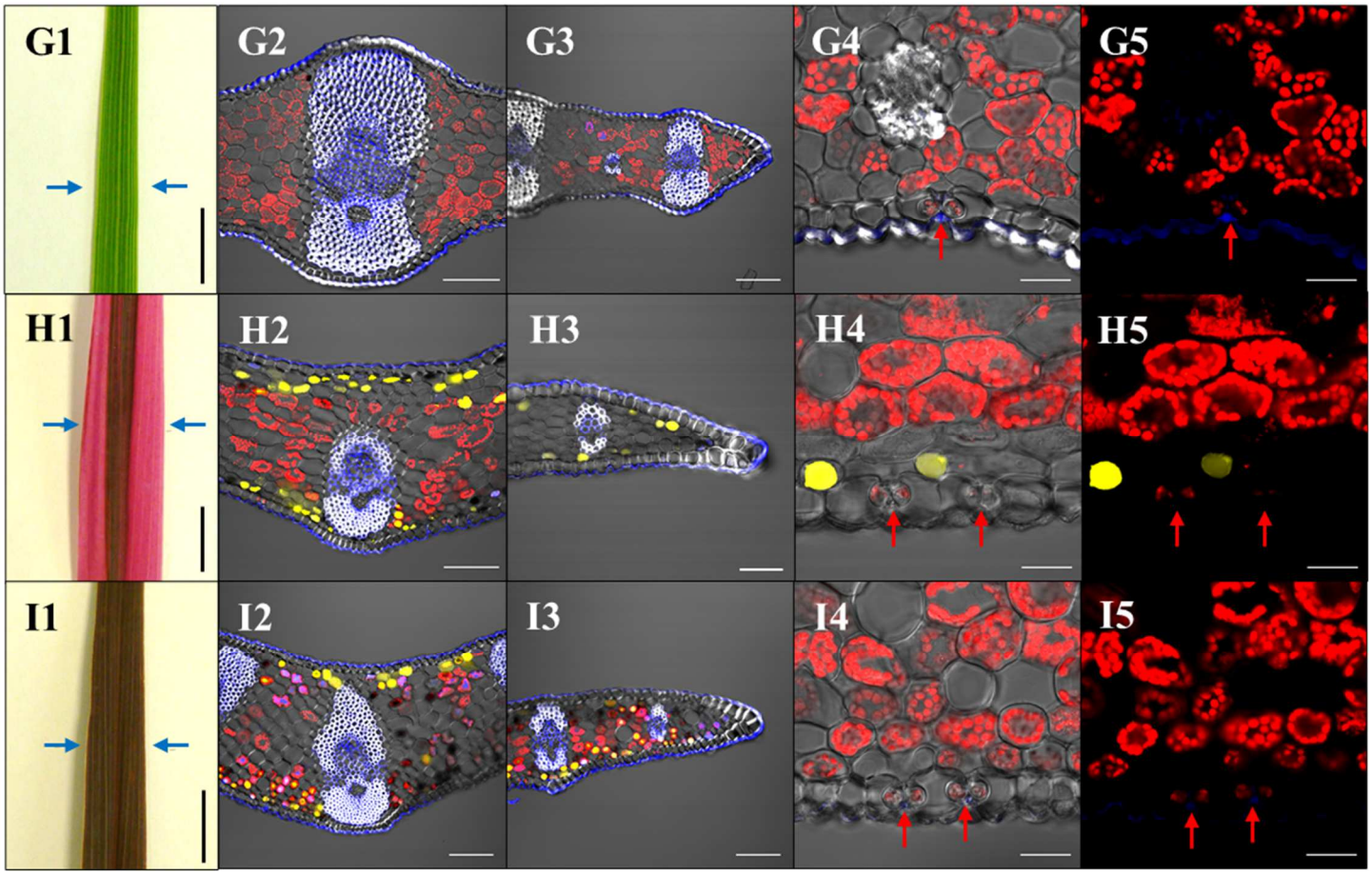


**Fig. 6**

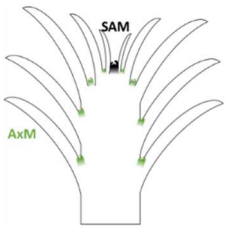
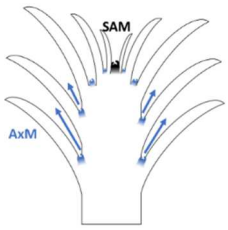
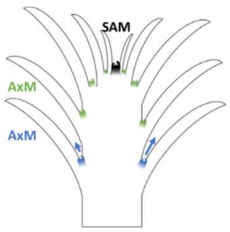
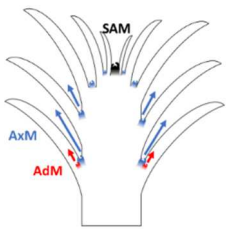
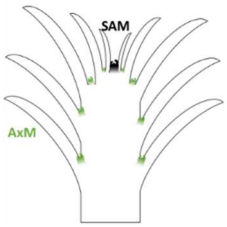
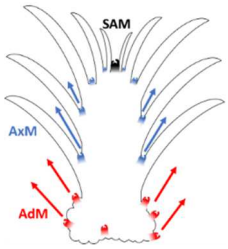
| Species | *In vitro* behavior on Control medium | *In vitro* behavior on medium with BAP |
|---|---|---|
| *Yucca gloriosa* |  |  |
| *Phormium tenax* |  |  |
| *Cordyline australis* |  |  |

**Fig. 7**

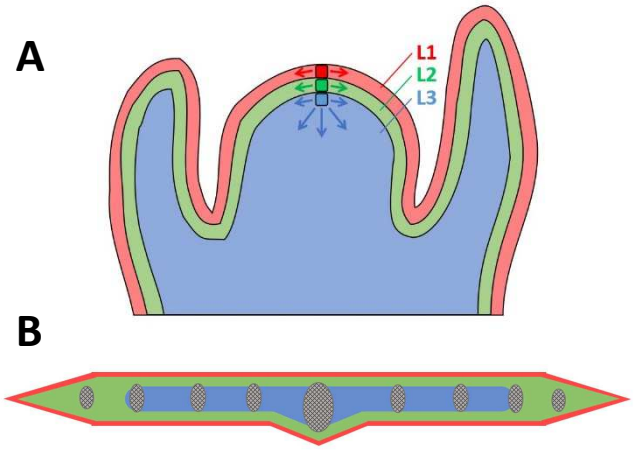


**Fig. 8**

| Genotype / Phenotype | Candidate pattern for meristem contribution to leaf development and chimeric structure |
|---|---|
| Yf WT | G-G-G |
| Yg VAR TTT | W-G-G |
| Yg VAR OT | W-W-G |
| Pt WT | G-G-G |
| PtJE TTT | G-G-W |
| PtJE OT | G-G-G |
| Ca WT | G-G-G |
| CaPP TTT | G-W-G |
| CaPP OT | G-G-G |
| CaPP OT | G-G-W |

## Table 1

**A.** Multiplication rate of ***Yucca gloriosa*** **'Variegata' (YgVAR)**

| | | BAP | | | | NPA | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub-culture | Shoot generat. | No. plants | No. dead | Means ± se | | No. plants | No. dead | Means ± se | | No. plants | No. dead | Means ± se | |
| M1 | SG0[a] | 47 | 3 | 1.14 ± 0.05 | c | 46 | 1 | 1.29 ± 0.08 | bc | 35 | 1 | 1 ± 0 | c |
| | **Tot M1[b]** | **47** | **3** | **1.14 ± 0.05** | **E** | **46** | **1** | **1.29 ± 0.08** | **DE** | **35** | **1** | **1 ± 0** | **E** |
| M2 | SG0 | 44 | 1 | 2.16 ± 0.14 | abc | 45 | 1 | 2.93 ± 0.16 | abc | 34 | 2 | 1 ± 0 | c |
| | SG1 | 6 | - | 1 ± 0 | c | 14 | - | 1.36 ± 0.17 | bc | - | - | - | - |
| | **Tot M2** | **50** | **1** | **2.02 ± 0.14** | **CD** | **59** | **1** | **2.55 ± 0.16** | **ABC** | **34** | **2** | **1 ± 0** | **E** |
| M3 | SG0 | 43 | 1 | 3.21 ± 0.19 | abc | 44 | - | 3.43 ± 0.14 | abc | 32 | - | 1 ± 0 | c |
| | SG1 | 6 | - | 2 ± 0.37 | abc | 14 | - | 2.57 ± 0.25 | abc | - | - | - | - |
| | SG2 | 51 | - | 1.53 ± 0.10 | bc | 90 | - | 1.84 ± 0.08 | abc | - | - | - | - |
| | **Tot M3** | **100** | **1** | **2.27 ± 0.13** | **BC** | **148** | **-** | **2.39 ± 0.09** | **BC** | **32** | **-** | **1 ± 0** | **E** |
| M4 | SG0 | 10 | - | 3.7 ± 0.21 | ab | 10 | - | 3.3 ± 0.34 | abc | 15 | - | 1 ± 0 | c |
| | SG1 | 2 | - | 3.5 ± 0.5 | abc | 5 | 1 | 1.6 ± 0.4 | abc | - | - | - | - |
| | SG2 | 10 | - | 2.7 ± 0.3 | abc | 19 | - | 2.37 ± 0.19 | abc | - | - | - | - |
| | SG3 | 28 | - | 2.43 ± 0.16 | abc | 44 | - | 2.02 ± 0.12 | abc | - | - | - | - |
| | **Tot M4** | **50** | **-** | **2.78 ± 0.14** | **AB** | **78** | **1** | **2.24 ± 0.11** | **BC** | **15** | **-** | **1 ± 0** | **E** |
| M5 | SG0 | 10 | - | 2.8 ± 0.49 | abc | 10 | - | 2.8 ± 0.25 | abc | 15 | - | 1 ± 0 | c |
| | SG1 | 2 | - | 2.5 ± 0.5 | abc | 4 | 1 | 3.33 ± 0.33 | abc | - | - | - | - |
| | SG2 | 4 | - | 3.25 ± 0.48 | abc | 6 | - | 3 ± 0.37 | abc | - | - | - | - |
| | SG3 | 15 | - | 2.6 ± 0.16 | abc | 21 | 1 | 2.95 ± 0.26 | abc | - | - | - | - |
| | SG4 | 49 | - | 2.43 ± 0.16 | abc | 48 | - | 2.94 ± 0.16 | abc | - | - | - | - |
| | **Tot M5** | **80** | **-** | **2.55 ± 0.12** | **ABC** | **89** | **2** | **2.94 ± 0.11** | **A** | **15** | **-** | **1 ± 0** | **E** |
| M6 | SG0 | 10 | - | 2.6 ± 0.22 | abc | 10 | - | 3.7 ± 0.34 | ab | 15 | - | 1 ± 0 | c |
| | SG1 | 2 | - | 4 ± 1 | ab | 3 | - | 3.33 ± 0.33 | abc | - | - | - | - |
| | SG2 | 2 | - | 3.5 ± 0.5 | abc | 6 | - | 3.17 ± 0.31 | abc | - | - | - | - |
| | SG3 | 9 | - | 2.89 ± 0.35 | abc | 20 | 1 | 3.37 ± 0.27 | abc | - | - | - | - |
| | SG4 | 30 | - | 3.17 ± 0.19 | abc | 48 | 1 | 2.89 ± 0.11 | abc | - | - | - | - |
| | SG5 | 74 | - | 2.46 ± 0.11 | abc | 97 | - | 2.64 ± 0.10 | abc | - | - | - | - |
| | **Tot M6** | **127** | **-** | **2.71 ± 0.09** | **AB** | **184** | **2** | **2.87 ± 0.07** | **A** | **15** | **-** | **1 ± 0** | **E** |
| E1 | SG0 | 10 | - | 2.3 ± 0.3 | abc | 10 | - | 2.6 ± 0.31 | abc | 15 | - | 1 ± 0 | c |
| | SG1 | 2 | - | 4.5 ± 1.5 | a | 3 | - | 2.67 ± 0.88 | abc | - | - | - | - |
| | SG2 | 2 | - | 3 ± 2 | abc | 6 | - | 3.67 ± 0.42 | ab | - | - | - | - |
| | SG3 | 9 | - | 2.33 ± 0.29 | abc | 19 | 1 | 3.06 ± 0.21 | abc | - | - | - | - |
| | SG4 | 30 | - | 3.13 ± 0.23 | abc | 47 | 1 | 3.11 ± 0.12 | abc | - | - | - | - |
| | SG5 | 74 | - | 2.68 ± 0.11 | abc | 97 | 2 | 3.4 ± 0.11 | abc | - | - | - | - |
| | SG6 | 112 | - | 1.97 ± 0.08 | abc | 178 | - | 2.33 ± 0.08 | abc | - | - | - | - |
| | **Tot E1** | **239** | **-** | **2.39 ± 0.07** | **BC** | **360** | **4** | **2.79 ± 0.06** | **A** | **15** | **-** | **1 ± 0** | **E** |
| **Total Medium[c]** | | **693** | **5** | **2.37 ± 0.04** | **B** | **964** | **11** | **2.63 ± 0.04** | **A** | **161** | **3** | **1 ± 0** | **C** |

***B.*** Multiplication rate of ***Phormium tenax*** **'Jessie' (PtJE)**

| | | BAP | | | | NPA | | | | Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub-culture | Shoot gen. | No. plants | No. dead | Means ± se | | No. plants | No. dead | Means ± se | | No. plants | No. dead | Means ± se | |
| M1 | SG0[a] | 61 | 2 | 1 ± 0 | i | 60 | 1 | 1 ± 0 | i | 44 | - | 1 ± 0 | i |
| | **Tot M1[b]** | **61** | **2** | **1 ± 0** | **F** | **60** | **1** | **1 ± 0** | **F** | **44** | **-** | **1 ± 0** | **F** |
| M2 | SG0 | 59 | 1 | 1.5 ± 0.08 | de-hi | 59 | 17 | 1.43 ± 0.08 | efghi | 44 | 4 | 1.03 ± 0.03 | i |
| | SG1 | - | - | - | - | - | - | | | - | - | - | - |
| | **Tot M2** | **59** | **1** | **1.5 ± 0.08** | **CDE** | **59** | **17** | **1.43 ± 0.08** | **C-F** | **44** | **4** | **1.03 ± 0.03** | **EF** |
| M3 | SG0 | 58 | 1 | 2.40 ± 0.15 | abc | 42 | 4 | 2.68 ± 0.16 | a | 40 | 1 | 1.03 ± 0.03 | i |
| | SG1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | SG2 | 30 | - | 1.07 ± 0.05 | i | 18 | 1 | 1 ± 0 | i | 1 | - | 1 ± NA | i |
| | **Tot M3** | **88** | **1** | **1.94 ± 0.12** | **AB** | **60** | **5** | **2.16 ± 0.15** | **A** | **41** | **1** | **1.03 ± 0.03** | **EF** |
| M4 | SG0 | 12 | - | 2.21 ± 0.11 | abcd | 12 | - | 2.29 ± 0.13 | abcd | 15 | - | 1 ± 0 | i |
| | SG1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | SG2 | 12 | - | 1.27 ± 0.08 | ghi | 12 | - | 1.24 ± 0.14 | ghi | 1 | - | 1 ± NA | i |
| | SG3 | 20 | - | 1.18 ± 0.06 | hi | 18 | - | 1.08 ± 0.04 | i | 1 | - | 1 ± NA | i |
| | **Tot M4** | **44** | **-** | **1.54 ± 0.06** | **CD** | **42** | **-** | **1.49 ± 0.07** | **CDE** | **17** | **-** | **1 ± 0** | **EF** |
| M5 | SG0 | 12 | - | 2.83 ± 0.30 | a | 12 | - | 2.58 ± 0.19 | ab | 15 | - | 1.13 ± 0.13 | hi |
| | SG1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | SG2 | 12 | - | 2 ± 0.25 | ab-fg | 12 | - | 1.92 ± 0.29 | ab-fg | 1 | - | 1 ± NA | i |
| | SG3 | 20 | - | 1.5 ± 0.17 | de-hi | 18 | 2 | 1.44 ± 0.18 | de-hi | 1 | - | 1 ± NA | i |
| | SG4 | 31 | 1 | 1.07 ± 0.05 | i | 26 | 2 | 1.08 ± 0.06 | hi | - | - | - | - |
| | **Tot M5** | **75** | **1** | **1.62 ± 0.11** | **BCD** | **68** | **4** | **1.61 ± 0.11** | **BCD** | **17** | **-** | **1.12 ± 0.12** | **DEF** |
| M6 | SG0 | 12 | - | 1.83 ± 0.24 | bc-gh | 9 | 1 | 2.33 ± 0.29 | abcd | 15 | - | 1 ± 0 | i |
| | SG1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | SG2 | 12 | - | 1.75 ± 0.25 | bc-hi | 12 | - | 2 ± 0.30 | ab-fg | 1 | - | 1 ± NA | i |
| | SG3 | 20 | 1 | 1.68 ± 0.19 | cd-hi | 16 | - | 1.69 ± 0.20 | bc-hi | 1 | - | 1 ± NA | i |
| | SG4 | 30 | - | 1.33 ± 0.13 | fghi | 24 | - | 1.88 ± 0.20 | bc-fg | - | - | - | - |
| | SG5 | 46 | 1 | 1 ± 0 | i | 36 | - | 1.14 ± 0.07 | hi | 2 | - | 1 ± 0 | i |
| | **Tot M6** | **120** | **2** | **1.36 ± 0.06** | **DEF** | **97** | **-** | **1.63 ± 0.09** | **BCD** | **19** | **-** | **1 ± 0** | **F** |
| E1 | SG0 | 9 | - | 1.78 ± 0.22 | bc-hi | 8 | - | 1.63 ± 0.18 | cd-hi | 12 | - | 1.58 ± 0.23 | cd-hi |
| | SG1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | SG2 | 12 | - | 2.17 ± 0.27 | ab-de | 12 | - | 2.08 ± 0.26 | ab-ef | 1 | - | 1 ± NA | i |
| | SG3 | 19 | - | 2.63 ± 0.23 | a | 16 | - | 2.44 ± 0.30 | abc | - | - | - | - |
| | SG4 | 30 | - | 2.53 ± 0.29 | ab | 24 | - | 2.58 ± 0.21 | ab | - | - | - | - |
| | SG5 | 42 | - | 1.36 ± 0.10 | fghi | 36 | - | 1.47 ± 0.12 | de-hi | 2 | - | 1 ± 0 | i |
| | SG6 | 42 | - | 1.12 ± 0.07 | hi | 62 | 1 | 1.28 ± 0.07 | ghi | - | - | - | - |
| | **Tot E1** | **154** | **-** | **1.77 ± 0.09** | **BC** | **158** | **1** | **1.72 ± 0.07** | **BC** | **15** | **-** | **1.47 ± 0.19** | **C-F** |
| E2 | SG0 | 9 | - | 1 ± 0 | i | 8 | - | 1 ± 0 | i | 12 | - | 1 ± 0 | i |
| | SG1 | - | - | - | - | - | - | - | - | - | - | - | - |
| | SG2 | 12 | - | 1 ± 0 | i | 12 | - | 1 ± 0 | i | 1 | - | 1 ± NA | i |
| | SG3 | 19 | - | 1 ± 0 | i | 16 | - | 1 ± 0 | i | - | - | - | - |
| | SG4 | 30 | 1 | 1 ± 0 | i | 24 | - | 1 ± 0 | i | - | - | - | - |
| | SG5 | 42 | - | 1 ± 0 | i | 36 | 1 | 1 ± 0 | i | 2 | - | 1 ± 0 | i |
| | SG6 | 42 | - | 1 ± 0 | i | 61 | - | 1 ± 0 | i | - | - | - | - |
| | SG7 | 122 | - | 1 ± 0 | i | 113 | - | 1 ± 0 | i | 7 | - | 1 ± 0 | i |
| | **Tot E2** | **276** | **1** | **1 ± 0** | **F** | **270** | **1** | **1 ± 0** | **F** | **22** | **-** | **1 ± 0** | **F** |
| **Total Medium[c]** | | **877** | **8** | **1.41 ± 0.03** | **A** | **814** | **29** | **1.41 ± 0.03** | **A** | **219** | **5** | **1.05 ± 0.02** | **B** |

***C.*** Multiplication rate of ***Cordyline australis*** **'Pink Passion'**

| | | **BAP** | | | | **NPA** | | | | **Control** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sub-culture | Shoot gen. | No. plants | No. dead | Means ± se | | No. plants | No. dead | Means ± se | | No. plants | No. dead | Means ± se | |
| M1 | SG0[a] | 38 | 3 | 1 ± 0 | b | 38 | 2 | 1.03 ± 0.03 | b | 32 | 1 | 1 ± 0 | b |
| | **Tot M1[b]** | **38** | **3** | **1 ± 0** | **E** | **38** | **2** | **1.03 ± 0.03** | **E** | **32** | **1** | **1 ± 0** | **E** |
| M2 | SG0 | 35 | 1 | 1.29 ± 0.08 | ab | 36 | 8 | 1.75 ± 0.11 | ab | 31 | - | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | **Tot M2** | **35** | **1** | **1.29 ± 0.08** | **CDE** | **37** | **8** | **1.72 ± 0.11** | **BC** | **31** | **-** | **1 ± 0** | **E** |
| M3 | SG0 | 34 | 11 | 1.74 ± 0.09 | ab | 28 | 8 | 1.95 ± 0.11 | ab | 31 | 3 | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | SG2 | 13 | 6 | 1.14 ± 0.14 | ab | 22 | 6 | 1.25 ± 0.11 | ab | - | - | - | - |
| | **Tot M3** | **47** | **17** | **1.6 ± 0.09** | **BCD** | **51** | **14** | **1.62 ± 0.10** | **BCD** | **31** | **3** | **1 ± 0** | **E** |
| M4 | SG0 | 12 | 1 | 2.09 ± 0.21 | a | 12 | - | 1.75 ± 0.18 | ab | 16 | - | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | SG2 | 5 | 3 | 1 ± 0 | b | 10 | 2 | 1 ± 0 | b | - | - | - | - |
| | SG3 | 11 | 2 | 1.11 ± 0.11 | ab | 14 | 2 | 1.17 ± 0.11 | ab | - | - | - | - |
| | **Tot M4** | **28** | **6** | **1.59 ± 0.16** | **BCD** | **37** | **4** | **1.33 ± 0.09** | **CDE** | **16** | **-** | **1 ± 0** | **E** |
| M5 | SG0 | 11 | - | 1.18 ± 0.12 | ab | 12 | - | 1.5 ± 0.15 | ab | 16 | - | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | SG2 | 2 | - | 1 ± 0 | b | 8 | 3 | 1.4 ± 0.25 | ab | - | - | - | - |
| | SG3 | 9 | 5 | 1.25 ± 0.25 | ab | 12 | 5 | 1 ± 0 | b | - | - | - | - |
| | SG4 | 13 | 3 | 1.1 ± 0.1 | ab | 11 | 3 | 1 ± 0 | b | - | - | - | - |
| | **Tot M5** | **35** | **8** | **1.15 ± 0.07** | **DE** | **44** | **11** | **1.24 ± 0.08** | **CDE** | **16** | **-** | **1 ± 0** | **E** |
| M6 | SG0 | 8 | - | 1.5 ± 0.19 | ab | 9 | - | 1.44 ± 0.18 | ab | 13 | - | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | SG2 | 2 | 1 | 1 ± NA | b | 5 | 1 | 1.25 ± 0.25 | ab | - | - | - | - |
| | SG3 | 4 | 1 | 1 ± 0 | b | 7 | 2 | 1.8 ± 0.2 | ab | - | - | - | - |
| | SG4 | 10 | 5 | 1 ± 0 | b | 8 | - | 1.5 ± 0.19 | ab | - | - | - | - |
| | SG5 | 1 | - | 1 ± NA | b | 8 | - | 1.13 ± 0.13 | ab | - | - | - | - |
| | **Tot M6** | **25** | **7** | **1.22 ± 0.10** | **CDE** | **38** | **3** | **1.4 ± 0.08** | **CDE** | **13** | **-** | **1 ± 0** | **E** |
| E1 | SG0 | 8 | - | 1.88 ± 0.23 | ab | 9 | 2 | 1.14 ± 0.14 | ab | 13 | - | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | SG2 | 1 | - | 1 ± NA | b | 4 | - | 1 ± 0 | b | - | - | - | - |
| | SG3 | 3 | 3 | - | - | 5 | 1 | 1.25 ± 0.25 | ab | - | - | - | - |
| | SG4 | 5 | 1 | 1.25 ± 0.25 | ab | 8 | - | 1 ± 0 | b | - | - | - | - |
| | SG5 | 1 | - | 1 ± NA | b | 5 | - | 1 ± 0 | b | - | - | - | - |
| | SG6 | 4 | 1 | 1 ± 0 | b | 14 | - | 1 ± 0 | b | - | - | - | - |
| | **Tot E1** | **22** | **5** | **1.47 ± 0.15** | **CDE** | **46** | **3** | **1.05 ± 0.03** | **E** | **13** | **-** | **1 ± 0** | **E** |
| E2 | SG0 | 8 | 1 | 1.71 ± 0.36 | ab | 7 | 2 | 1.4 ± 0.25 | ab | 13 | - | 1 ± 0 | b |
| | SG1 | - | - | - | - | 1 | - | 1 ± NA | b | - | - | - | - |
| | SG2 | 1 | - | 1 ± NA | b | 4 | 1 | 2.67 ± 0.88 | a | - | - | - | - |
| | SG3 | - | - | - | - | 4 | - | 1.75 ± 0.25 | ab | - | - | - | - |
| | SG4 | 4 | 2 | 2.5 ± 0.5 | a | 8 | 2 | 3 ± 0.68 | a | - | - | - | - |
| | SG5 | 1 | - | 3 ± NA | a | 5 | - | 2 ± 0.45 | ab | - | - | - | - |
| | SG6 | 3 | - | 2 ± 0 | ab | 14 | 2 | 2 ± 0.28 | ab | - | - | - | - |
| | SG7 | 8 | 1 | 3 ± 0.69 | a | 2 | - | 1 ± 0 | b | - | - | - | - |
| | **Tot E2** | **25** | **4** | **2.29 ± 0.29** | **A** | **45** | **7** | **2.03 ± 0.18** | **AB** | **13** | **-** | **1 ± 0** | **E** |
| **Total Medium[c]** | | **255** | **51** | **1.41 ± 0.05** | **A** | **336** | **52** | **1.42 ±0.04** | **A** | **165** | **4** | **1 ± 0** | **B** |

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**Table 2**

| Cultivars | | From true-to-type parent[a] | | From off-type parent[b] | | |
|---|---|---|---|---|---|---|
| | **Number of plants** | **True-to-type plants (%)[a]** | **Off-type plants (%)[b]** | **True-to-type plants (%)[a]** | **Off-type plants (%)[b]** | **Chi² Test (p-value)** |
| *Yucca* 'Variegata'[c] | 2641 | 2417 (95.7%) | 108 (4.3%) | 41 (35.3%) | 75 (64.7%) | 2.2e-16 |
| *Phormium* 'Jessie'[d] | 478 | 340 (75.4%) | 111 (24.6%) | 13 (48.1%) | 14 (51.9%) | 0.003694 |
| *Cordyline* 'Pink Passion'[c] | 148 | 63 (60.6%) | 41 (39.4%) | 0 (0%) | 44 (100%) | 3.344e-11 |