RESEARCH ARTICLE

Shoot organogenesis and pluripotency profile in daylily whole flower bud

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This study was designed to evaluate the potential application of whole flower bud as explant for *in vitro* plant regeneration and attempt to profile potentially natural distribution of cellular totipotency or pluripotency in the organ using Murashige and Skoog nutrients medium with thidiazuron and kinetin growth regulators. All cultures were incubated under room environmental conditions. The results were promising, because it demonstrated a possible inherent shoot organogenic pattern in the perianth that reflected potential cellular pluripotency that trended decreasingly from the ovary. It also confirmed the study's hypothesis that the whole flower bud could be applied as a reliable explant for consistent and effective multiple shoot inductions in less than 30 days. The results could, overall, expedite development of more efficient and consistent protocols for daylily floral organ applications and inspire similar research in other plant species.

Keywords: Plant regeneration; daylily; Hemerocallis; tissue culture; shoot organogenesis; micropropagation; totipotency; pluripotency; ovary; caulogenesis; callogenesis.

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Introduction

The daylily industry thrives on phenotypic diversity, which also typically reflects genotypic variations. However, considering that the constancy of lucrative phenotypes is required, sexual reproduction is not a preferred method [1-3] for daylily commercial multiplication. Furthermore, based on general daylily breeding strategies and practices [4-8], most daylily cultivars are not bred true. Micropropagation is potentially and arguably the fastest and most effective *in vitro* approach for reproducing the plant parent identity in progenies [9, 10] and gene transfer for crop improvement [11-13]. Although the flower dip approach [14, 15] has been proposed as an alternative to micro-propagation method for genetic transformation, it is practically preferred for experimental studies and inefficient for largescale commercial applications. Moreover, floral dip transformation still requires sexual reproduction to produce seeds, and thus, the progenies are more susceptible to genetic variations. Therefore, micropropagation methods are the most practical and reliable strategies.

The relevancy of micropropagation is the recognition of assertive cellular totipotency and pluripotency in applicable tissues under special

culture conditions [16, 17]. Here, individual plant cells are competent of multiplying and specializing into different functions to make up a whole new organism. This cellular capacity, totipotency, has been established in daylilies [18, 19]. Similarly, its derivative concept, pluripotency [20-23], which delineates cellular potential to develop into more than one types of organs (shoots, roots, or flowers), has also been evidenced in daylily tissue culture [24-26]. Considering the difficulty of micro-propagating daylilies *in vitro* consistently, it is important to assess the potential for cellular totipotency and/or pluripotency in floral buds to determine whether there might exist an inherent pattern that might be explored for developing more consistent and efficient tissue culture protocols. Flower buds are a daylily's most studied organ for *in vitro* plant regeneration [27-30], probably, because it is the only organ that can yield a spectrum of sub-explant types (sepal, petal, ovary, filament, ovule, style, anther, pollen, and receptacle) compared to other organs (leaf, root, and stem); or perhaps because this organ has responded more positively in tissue culture compared to other daylily explants evaluated. Irrespective of the reason, it will be more informative to accurately reflect on the pattern of the floral totipotency or pluripotency occurrence. The understanding of it will not only improve daylily *in vitro* plant regeneration protocols, but also make it more accessible for genetic improvement using modern technologies [13, 31-33]. This study attempted to determine whether there exists an inherent pattern of adventitious plant regenerative responses within the flower bud that might reflect potential cellular totipotency and/or pluripotency gradient that could be beneficial in advancing and broadening daylily tissue culture and genetic improvement applications.

Material and methods

Plant materials

The cultivars that were studied were field-grown and included "Intricate Art", "Empire State", "Tail Feather", "Creepy Crawlers", "Siloam Virginia Henson", "Orange Slices", "Coyote Moon", "Rococo", "Grape Velvet", "Gay Hearted", "Science Stealer", "Bright Banner", and "Alias" species.

Explant preparation and cultural conditions

Young buds (0.5-2.5 cm long) (Figure 1) were freshly collected and surface-sterilized with 35% sodium hypochlorite bleach (commercial Clorox) for 10 minutes; then, rinsed four times with sterile distilled water. Five whole sterile bud explants were randomly assigned to individual treatments, as replicates, and cultured one bud per test tube. As buds grew bigger, they were transferred to Magenta 7 (GA-7) containers (Magenta Corporation, Lockport, IL, USA) on the same cultural medium. Five additional buds were cross-sectioned and used as control explants. However, all pieces of individual control buds were cultured on a single Petri dish to ensure that the natural order or polarity of those pieces in the organ was preserved. The nutrients medium consisted of Murashige and Skoog (MS) salts and vitamins [34], sucrose (20 g/L), and kinetin (0, 1, and 5 mg/L) and/or (0, and 1 mg/L) thidiazuron (TDZ). Kinetin or TDZ were used alone or in combination and the nutrient medium without growth regulators (MS0) was used as the control medium. All nutrients medium containers with explants were sealed with parafilm. Each explant was used as an experimental unit for observations and data collection. The final pH of the medium was adjusted to 5.8 with 1 M NaOH after the addition of phytagel (4 g/L). The media were autoclaved at 121°C for 20 min. Explants were sub-cultured onto fresh media every four weeks. After reaching at least 2 inches long, newly induced individual shoots were separated and sub-cultured on MS0 for inducing roots. Sufficiently rooted shoots (at least five roots of at least 10 centimeters with abundant root hairs) were transferred to potted soil for 1 month-acclimation and 3-5 months in the greenhouse prior to transferring to the field. All chemicals used in this experiment were purchased from Sigma (St. Louis, MO, USA). All culture containers were incubated at 8-hour photoperiod at room

Figure 1. Fresh floral organs including buds, whole flowers, and ovary at the base of pistil after removing corolla and calyx.

environmental conditions. During the culture, the room temperature varied from 15.6°C to 36.6°C, and the humidity from 21% to 55%. The light sources were regular fluorescent tubes (GE 10773, 60Watt, 48 Inch, T12 Linear Fluorescent, 4100K, 60 CRI, Recessed Double Contact (R17D) Base, High Output Tube (F48T12/CW/HO/GE)).

Experimental observations and data collection

Experimental observations and data collection on culture responses were made daily on individual dishes/GA-7/test tubes for 85 days of experimental cut. The results encompassed only data collected from the original explants, within the experimental period. During the experiment, we used a Stereomaster binocular light microscope (Thermo Fisher Scientific, Waltham, MA USA) for counting shoot primordia and shoot buds and shoots. A standard AT&T GoPhone photo camera was applied for pictures.

Statistical analysis

The study was carried out using the randomized factorial design. Data were analyzed by using the linear models with interactions among factors. The analyses were performed with R and RStudio software (version 3.6, 2019) (Boston, MA, USA). The statistical package used for analysis of variance was emmeans [35] and that for plotting and graphs was ggplot2 [36]. The significance of mean differences was tested by using the Tukey Test at 5% level.

Results

Multiple shoot organogenesis was successfully and consistently induced in the whole flower bud explants that were cultured *in vitro* during the 85-day-study period. With a few exceptions, shoot organogenesis occurred in a pattern that reflected a gradient of cellular pluripotency trending decreasingly from the ovary.

Profile of shoot organogenic pluripotency

Following whole flower bud explants culture, shoot organogenesis was observed in the ovaries (Figure 2). Here, the ovarian region underwent

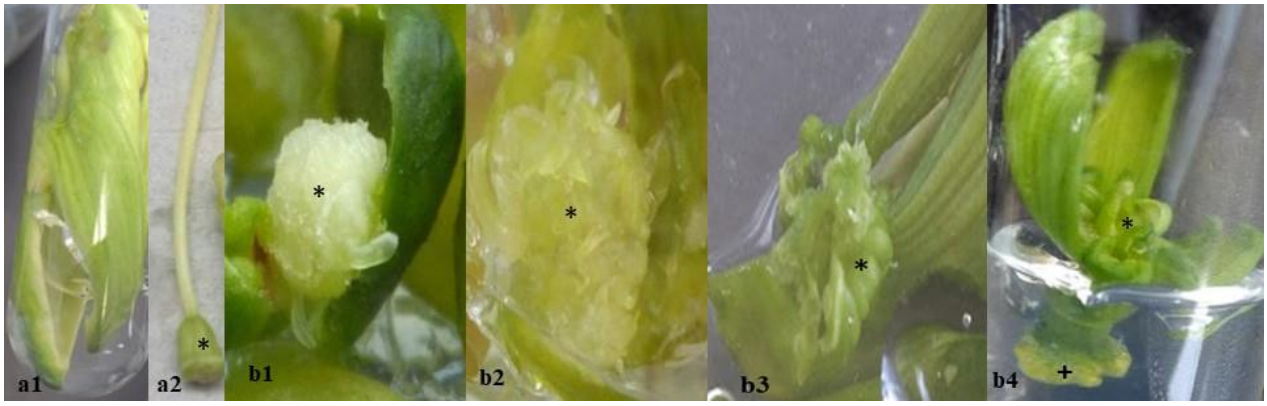

Figure 2. Flower bud ovary (*) developmental shoot organogenesis. **a1:** 40-day-old non-responsive control bud cultured on MS0 medium. **a2:** freshly collected control ovary. **b1:** 22-day-old ovarian early shoot organogenesis. **b2:** 30-day-old ovarian mid shoot organogenesis. **b3:** 38-day-old ovarian advanced shoot organogenesis. **b4:** 48-day-old bid showing ovarian shootlets (*) and primal shoot organogenesis at receptacle lower extremity (+).

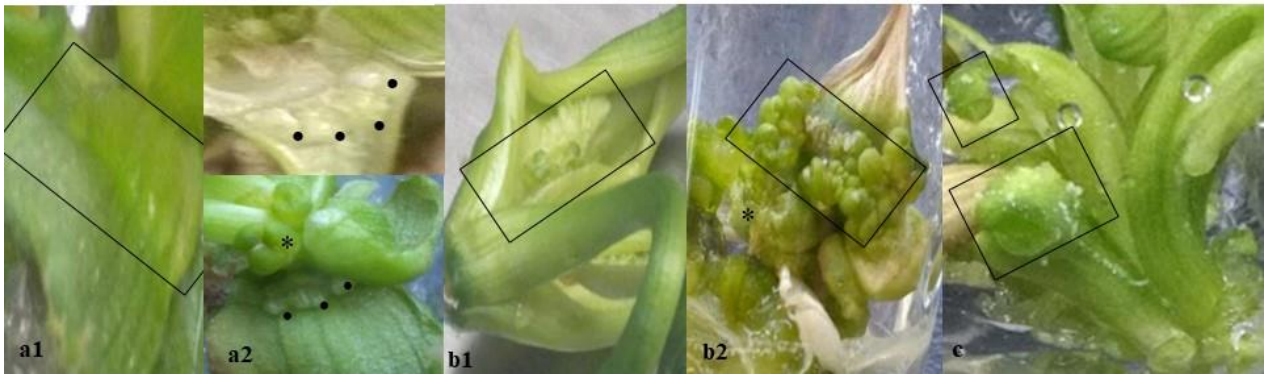

Figure 3. Developmental shoot organogenesis in petal/sepal regions (basal, middle, and terminal). **a1:** 13-day-old broad swelling (boxed section). **a2:** 13-day-old localized bulges (dots) of primordial shoot organogenesis in the perianth (petal/sepal) base and ovarian shoot buds (*). **b1:** 48-day-old mid development. **b2:** 68-day-old advanced development of shoot organogenesis in basal through middle regions of the perianth (boxed section) and ovarian shoot buds (*). **c:** 63-day-old petal/sepal terminal shoot organogenesis (boxed sections).

massive mitoses that resulted in a swollen dome-like structure several folds larger (Figure 2, b1 and b2) than the original size (Figure 2, a2), and subsequently induced abundant multiple shoots. With a few exceptions, when massive shoot organogenesis occurred in the ovary, organogenic responses seemed to be inhibited in the rest of the other parts of the whole flower bud explant. Accordingly, no specific cultural variables were associated with such a response. In those exceptions, dual little to moderate shoot organogenic activities occurred in both the ovary and lower extremity of the receptacle (Figure 2, b4) or perianth (Figure 3, a2 and b2) of the whole buds. With independent focus on the perianth (petals and/or sepals), when shoot organogenesis occurred in petals/sepals, it was preceded by either broad basal swelling (Figure 3, a1) or localized bulges (Figure 3, a2) that subsequently developed into shoot buds and shoots. In this case, the occurrence of shoot organogenesis expanded from the base through the middle regions of responding petals/sepals (Figure 3, b1 and b2) with generally no response in the terminal region. However, a few exceptions in which limited shoot organogenic activities restricted to the terminal region (Figure 3, c) that did not result in successful shoot development were observed. Alternatively, shoot organogenic responses occurred

Figure 4. Developmental shoot organogenesis in the receptacle lower extremity (*) of the whole flower bud. **a1:** 7-day-old primal organogenesis. **a2:** 25-day-old shoot buds. **a3:** 38-day-old shootlets. **b:** 40-day-old sectioned bud explants (control explants) (•: shoot organogenesis in the ovarian region explants. +: shoot organogenesis in the perianth basal region explants).

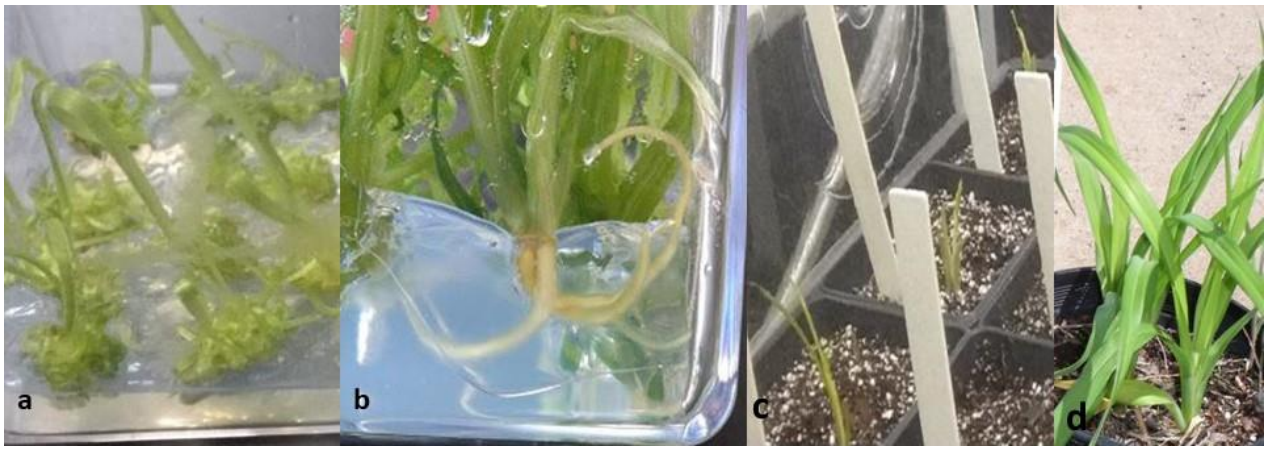

Figure 5. a: 55-day-old cultures of multiple shoots clusters that were split from an original explant for multiplication and elongation on shoot inducing medium. **b:** 70-day-old normally growing rooted shoots on MS0. **c:** 76-day-old transplants acclimation. **d:** potted plants ready for field transplanting.

restrictively in the receptacle's lower extremity of the bud explants (Figure 4, a1-a3). In this case, with a few exceptions, there were no or no significant organogenic responses that occurred in the ovaries. Observations of the control explants that were cross-sectioned showed shoot organogenesis occurring mostly in the ovary and, occasionally, in the petal/sepal basal region (Figure 4, b). However, no positive responses were observed in the middle or terminal region of responding petals/sepals.

Generally, when multiple shoot buds or shootlets were sub-cultured in clusters on the same shoot inducing medium, they multiply and elongated faster prior to separating for rooting and acclimation and transferring into the greenhouse (Figure 5). All plants grew healthy normally.

Global shoot organogenic responses

Shoot organogenesis was variably observed in all varieties that were studied, and statistical data are presented in Figures 6 and 7. Overall, 54% of the varieties studied formed shoots across all treatments (Figure 6). Although the other 46% of varieties performed also generally well, they all failed to induce shoot organogenesis in at least one treatment. Furthermore, all varieties

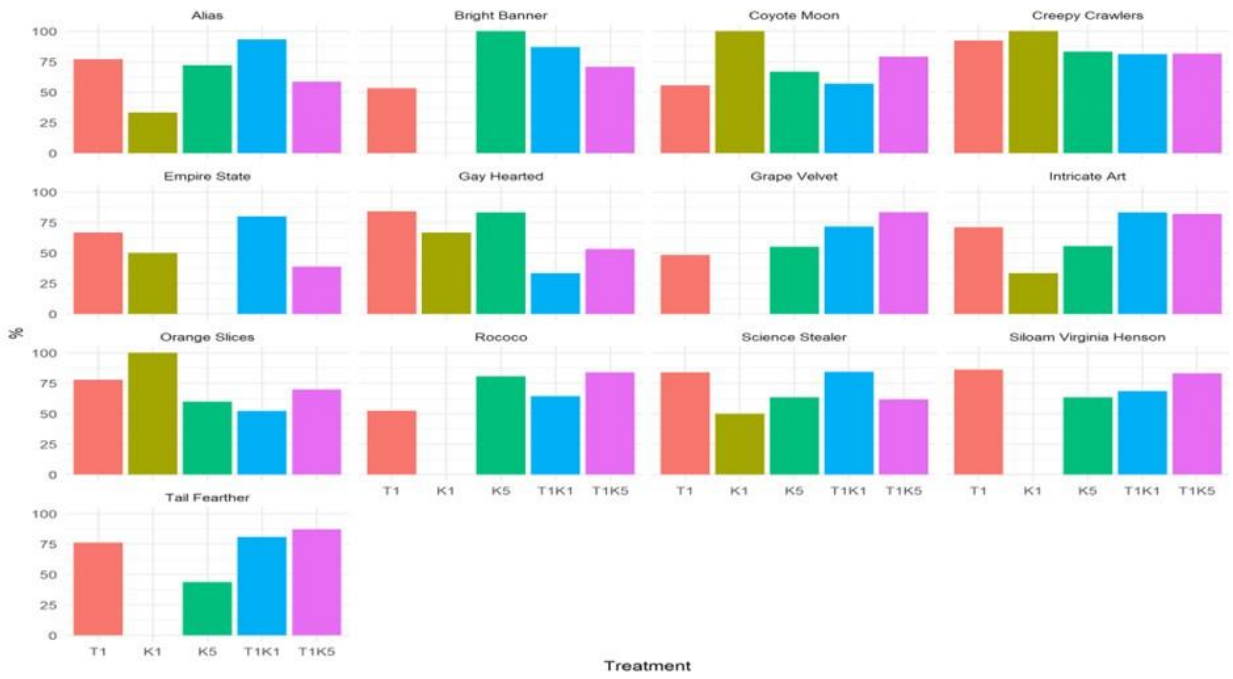

Figure 6. Varietal shoot organogenic responses to individual growth regulator treatments (kinetin and TDZ). **T1:** 1 mg/L TDZ. **K1:** 1 mg/L kinetin. **K5:** 5 mg/L kinetin. **T1K1:** combination of 1 mg/L TDZ and 1 mg/L kinetin. **T1K5:** combination of 1 mg/L TDZ and 5 mg/L kinetin. Five replicates per treatment. Mean differences were tested at 5% level of significance with Tukey Test using R and RStudio software. %: percent shoot organogenic responses per treatment per variety.

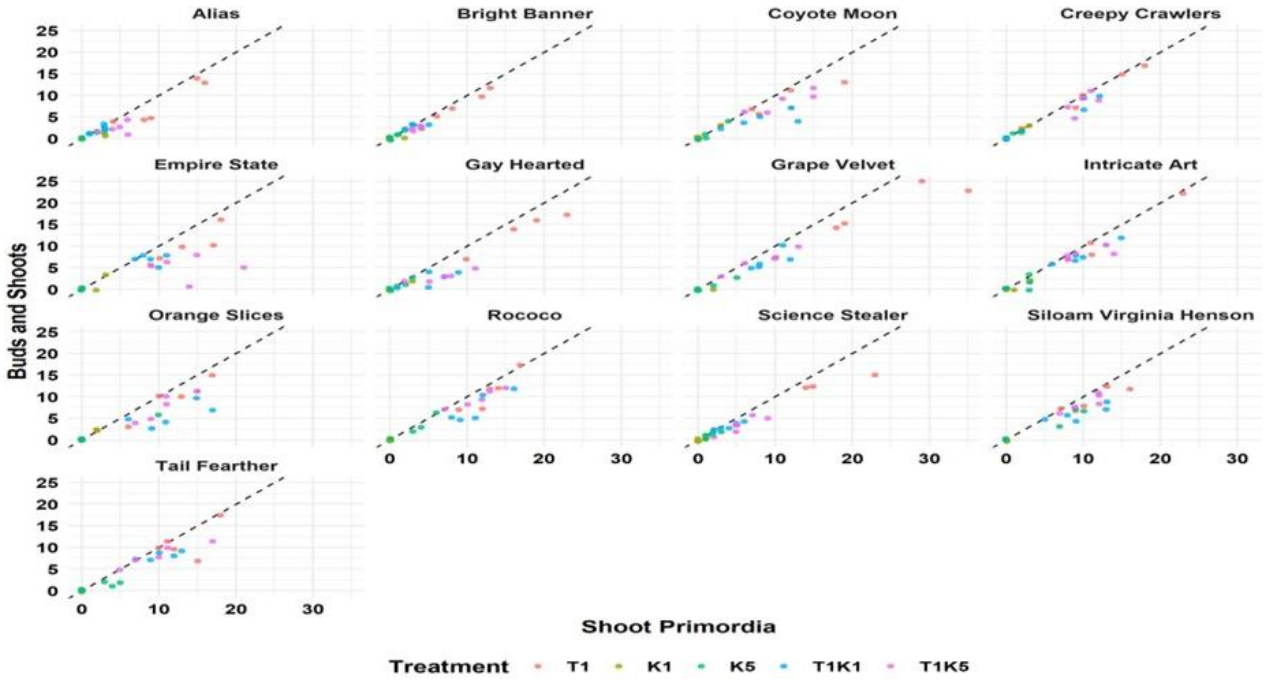

Figure 7. Varietal shoot organogenic responses (the amount of shoot primordia or buds and shoots per explant) to growth regulator treatments. **T1:** 1 mg/L TDZ. **K1:** 1 mg/L kinetin. **K5:** 5 mg/L kinetin. **T1K1:** combination of 1 mg/L TDZ and 1 mg/L kinetin. **T1K5:** combination of 1 mg/L TDZ and 5 mg/L kinetin. Five replicates per treatment. Mean differences were tested at 5% level of significance with Tukey Test using R and RStudio software.

induced at least 60% shoot organogenesis in at least two treatments, while four varieties including “Bright Banner”, “Coyote Moon”, “Creepy Crawlers”, and “Orange Slices” induced 100% shoot organogenesis in at least one treatment. The most ineffective treatment was 1 mg/L kinetin that failed to induce organogenesis in 38.5% of the varieties studied. The other limitedly less effective treatment was 5 mg/L kinetin, because of its failing to induce organogenesis in one variety, “Empire State”.

Individual varietal performances for *de novo* shoot induction *in vitro* were also overall encouraging (Figure 7). The top five shoot bud and shoot numbers per explant per variety that were observed included 25, 22.5, 17.5, 16, and 15 for the varieties of “Grape Velvet”, “Intricate Art”, “Creepy Crawlers”/“Rococo”/“Tail Feather”/“Gay Hearted”, “Empire State”, and “Orange Slice”/“Scene Stealer”, respectively. Accordingly, the top shoot bud and shoot averages per explant were also observed in those varieties including “Grape Velvet” (16.8), “Intricate Art” (11.6), “Rococo” and “Tail Feather” (11), “Gay Hearted” (10.8), and “Creepy Crawlers” (10.2). Most noticeably, all those top performances resulted from the 1 mg/L TDZ treatment. All combination treatments induced shoot organogenesis across all varieties (Figures 5-6). The top numbers of shoot buds and shoots, ranging from 10 to 12.5 per explant, were induced in nine varieties in response to the combination treatment 1 mg/L TDZ and 5 mg/L kinetin. Those varieties included “Rococo” and “Coyote Moon” (12.5), “Tail Feather” (12), “Creepy Crawlers” (11.3), “Siloam Virginia Henson” (11), and “Orange Slices”, “Tail Feather”, “Intricate Art” (10). On the other hand, the top shoot formations, from the combination treatment of 1 mg/L TDZ and kinetin, ranging from 9 to 12.5 shoot buds and shoots per explant, were observed in seven varieties including “Intricate Art” and “Rococo” (12.5), “Creepy Crawlers”, “Grape Velvet”, “Orange Slices”, “Tail Feather” (10), and “Siloam Virginia Henson” (9).

Discussion

Ovarian totipotency and pluripotency have been positively made full use of in other plant species [37]. In the present study, it was, overall, shown that the whole flower bud can be applied as an effective explant for *de novo in vitro* shoot induction (caulogenesis). Caulogenesis [38], which is a variant of pluripotency, occurred in this study directly as well as indirectly. The study also provides some insights about the potential for direct organogenic responses when the whole organ is cultured *in vitro*.

Since the demonstration of pluripotency in daylilies [24], several tissue cultures studies have confirmed it in cells of variable tissues such as leaf [25], stem [39], and inflorescence [28, 40-42]. However, this is the first time a study has applied the whole flower bud as an explant and focused on its response pattern to determine whether there exists a totipotency or pluripotency gradient; and the results were promising. Premising on the fundamental concept that morphogenesis is, generally, a stressful response to wounding [43-46], there was no expectation on what the whole bud’s response would be. Irrespective of the path for achieving shoot regeneration in the whole floral bud, there was clear evidence that ovary was the core of pluripotency and appeared also to be related to the occurrence of caulogenic activities in other proximal floral tissues.

The pioneering successful study that applied ovaries as explants in daylily *in vitro* tissue culture could be credited to Krikorian and Kann [39], who obtained multiple shoots via callus. Because they did not induce direct shoots in the ovaries, it was difficult to confidently assess the level of pluripotency in original tissue cells. However, their work showed the potential of using ovaries as an independent explant or source of pluripotent cells that built on the demonstrative studies that primarily induced callus in daylily ovaries by Mullin [47]. Another noticeable study that built on Krikorian and Kann’s report [40] was by Mahagamasekera [27]. In this study, the

author split the ovary lengthwise for explants and observed that, not only, shoots could form directly from explants, but also that the ovary explants induced the greatest numbers of shoots across treatments compared to other explant types that were used. Those results align with ours, in which the whole unsevered ovary was studied and showed that the ovary was the center of greater organogenic activities than other types of floral tissues of the same bud explant. More importantly, the study also showed that there may exist a link between organogenic responses in the ovary and perianth of the whole bud explant. For example, when massive shoot organogenic activities occurred in the ovary, it often seemed to prevent such a response in other bud's tissues. However, when zero to moderate shoot organogenesis occurred in the ovary, shoot organogenesis occurred in other flower bud tissues proximal to the ovary, such as petal, sepal, or receptacle.

When considering the response of perianth independently, it was clearer that shoot organogenesis developed in gradient decreasing from the base through the tip of the responding organ. The only exception was when the petal/sepal tips occasionally formed some shoot primordia that never materialized in shoot buds or shoots. Overall, when shoot organogenesis occurred in the perianth, there were greater response in the base than middle regions of petals/sepals. Although there is no specific explanation, it was thought that being the natural site for initiating the formation of the first cell of the new organism, zygotic cell, the ovary may exceptionally be prone to retaining greater cellular juvenility that may extend to proximal cells or tissues and influence the trending response that was observed in the perianth. Although daylily petals have previously been cultured in tissue culture for shoot organogenesis [48], all shoots were obtained via callus, which made it difficult to authentically assess totipotency or pluripotency for direct plant formation in the original explant cells. The present results corroborate findings by Mahagamasekera [27], in which the author used cross-sectioned daylily petals and sepals in three (base: equivalent of middle region in our study, middle and tip: equivalent of terminal in our study) or two (base to middle through tip) explant types, depending on the bud size, in addition to what was described as tube (equivalent of base in our study). In his study, Mahagamasekera observed that only basal and tube explants induced shoot organogenesis. No positive responses were observed in middle and terminal petal/sepal explants. Although the results gave the first direct indication of the possible existence of pluripotent gradient in petals/sepals, the effect of disrupting the tissues by splitting into separate explants was not certain, considering the positive effect of wounding on inducing organogenesis [49, 50]. Partitioning organs into smaller explant pieces in tissue culture is necessary to induce wound stress that triggers mitotic divisions intended primarily to heal wounds. However, in the process some of the cells acquire new differentiation and specialization capacities that result in adventitious organ formation [43, 49, 50]. Therefore, further investigations were needed, which inspired the current study. Coupling the present observations with that of Mahagamasekera, it is increasingly convincing that a pluripotency gradient may exist in daylily perianth tissues. However, further studies still are needed to determine required variables and standardize environmental conditions to enhance the understanding and maximize the applications.

Conclusion

This study reports a one-step protocol for caulogenesis in daylily whole flower bud and substantiates the hypothesis that pluripotency gradient might be inherent in the crop's floral organs. Accordingly, greater organogenic responses were generally observed in the basal than middle regions of the perianth, without a positive response in the terminal region of either sepals or petals. Furthermore, variable levels of caulagenesis were observed in the ovary and

seemed to be related to organogenic responses in other proximal floral tissues. These results could improve and broaden the applications of whole buds in daylily micropropagations as well as genetic improvement.

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