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In vitro daylily (*Hemerocallis* species) bract multiple shoot induction

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A bract is a non-inflorescence structure that exists in many plant species. In daylilies, the bract is biologically functional and is the object of the present tissue culture study. After sterilization, bract explants were treated with 6-benzylaminopurine (BA) and thidiazuron (TDZ) that were used individually or in combination in Murashige and Skoog (MS) nutrients medium, under room environmental conditions, to study its capacity to induce shoots *in vitro*. The results were successful. Both direct and indirect shoot organogenesis were observed. Although variably, all nineteen cultivars that were investigated induced multiple shoots. TDZ was the most potent chemical stimulus for shoot organogenesis. The results also showed no significant correlation between shoot conversion potential and genotype or treatment.

Key words: Daylily, tissue culture, plant organogenesis, bract, *Hemerocallis*, plant regeneration, thidiazuron.

INTRODUCTION

Daylilies are a monocot of great socio-economic and research values that thrive primarily because of its floral beauty and landscaping applications (Hansen, 2007; Rodriguez-Enriquez and Grant-Downton, 2013; Cui et al., 2019; Li et al., 2020). They are also increasingly justified for medicinal usage and pharmaceutical studies (Du et al., 2014; Farcas et al., 2019). Like many other crops of economic value, daylilies are explored for consistent *in vitro* mass multiplication for functional purposes. The application of *in vitro* micropropagation approaches is of a special consideration in daylilies, because the crop's most economically valued aspect (floral beauty) requires faithful reproduction of the parental phenotype that usually guides customers' choice. Accordingly, *in vitro* tissue culture is among the most effective approaches to reliably deliver customers' expectation in a timely manner and in large-scale. Despite some progress (Matand et al., 2020), daylilies remain very challenging for *in vitro* micropropagation, especially, *de novo* plant regeneration. Unfortunately, there is an incorrect perception that *in vitro* plant organogenesis is easily attainable in daylilies. This idea is often promoted by sporadic reports of protocols that are frequently inconsistent or difficult to reproduce. Consequently, it has hindered efforts to improve the crop

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Figure 1. Inflorescences on the field experiment plants. It also shows a cluster of flower buds with a display of bract samples.*Identifies non-sterile bract units.

utilizing contemporary technologies like genetic transformation and/or CRSPR/Cas9 editing. Therefore, it is important to expand efforts broadening the studies of various tissues or cell types for totipotency until efficient and convenient reproducible protocols are available for broad applications across *Hemerocallis* species.

This study evaluates bract cells' totipotency. The bract is a genetically inherent, non-floral structure that exists in many plant species (Bowman and Jones, 1982; Whipple et al., 2010; Chuck et al., 2010; Chandler, 2014). It performs vital functions ranging from attracting pollinators to protecting inflorescences (Luke, 1968; Rose, 2016). Its prominence in daylilies lends it the suitability of a potentially reliable explant for *in vitro* tissue culture applications. The understanding of its cellular totipotency could also lead to developing an alternative plant regeneration protocol *via* protoplasts, considering that bract tissues are soft and amenable to protoplasts generation which would be compatible, for example, with CRSPR/Case 9 technology.

MATERIALS AND METHODS

Plant genotypes

The cultivars that were studied included 'Atlanta Dove Child', 'Dark Star', 'Water Birds', 'Realms of Glory', 'Look', 'Atlanta Full House', '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'.

Explant preparation and cultural conditions

Young inflorescences were freshly collected from 19 cultivars of daylilies in the Langston University (Oklahoma, U.S.A) collection (Figure 1). These were sectioned into subunits and surface-sterilized with 35% sodium hypochlorite solution (commercial Clorox bleach) for 8 min; then, rinsed with sterile distilled water three times. The whole bract was used as explant and cultured individually in test tubes, one unit per test tube containing 5 ml of culture medium. When needed, the tip of the bract tissue was trimmed to ensure that the explant fits properly in the recipient tube. It was previously determined that trimming tips in this manner did not affect the explant response.

Overall, five test tubes with explants (five replicates) were randomly assigned to individual treatments. Each tube was applied as an experimental unit for observations. Three levels of 6-Benzylaminopurine (BA) (0, 1, and 3 mg/l) defined treatment groups, while 1 mg/l thidiazuron (TDZ) and 0 mg/l growth regulator culture media were used as a positive and negative control treatments, respectively. In total, the experiment applied 5 treatments. Treatments were used alone or in combination in the Murashige and Skoog (MS) nutrients medium. The MS medium consisted of MS salts and vitamins (Murashige and Skoog, 1962), 20 g/l sucrose, and 4 g/l phytagel. Cultures were transferred to fresh media monthly. After at least 45 days of culture, responding explants grew bigger and were sub-cultured into Magenta 7 (GA7) containers with corresponding treatments. All containers were wrapped with parafilm and incubated at 8-h photoperiod at room environmental conditions. During the culture, the room temperature varied from 18.3 to 35.5°C, and the humidity from 21 to 55%. The light sources were regular fluorescent tubes (GE 10773, 60 Watt,

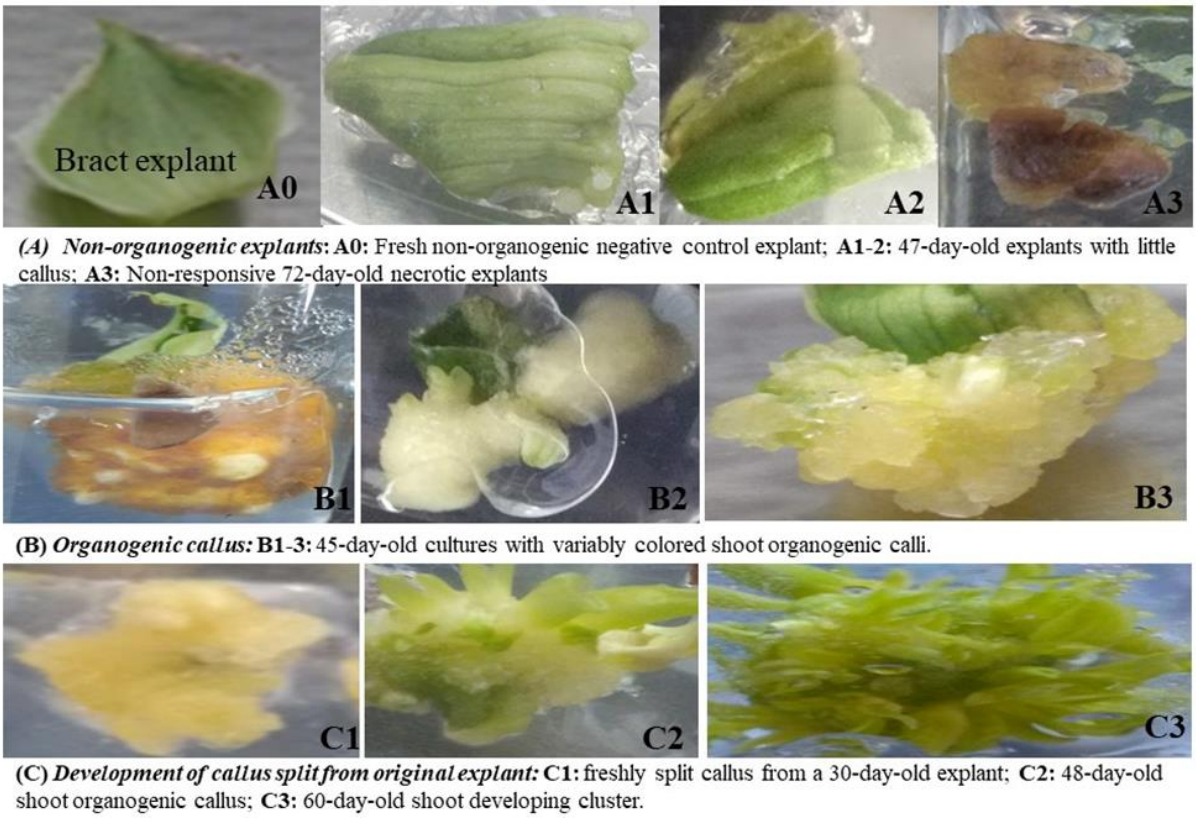

Figure 2. Bract morphogenesis showing variable callus and indirect shoot organogenesis.

48 Inch, T12 Linear Fluorescent, 4100K, 60 CRI) Base, High Output Tube (F48T12/CW/HO/GE)). The final pH of the medium was adjusted to 5.8 - 6.0. The media were autoclaved at 121°C for 20 min. All chemicals were purchased from Sigma Co. (St. Louis, MO, USA).

Data observations and collections

Observations and data collections were conducted daily on individual explants (experimental units) starting from the first week of culturing, for a ninety day-period. Observations included callus, shoot primordium, bud and shoot, and root inductions. Primordia and buds and shoots were counted on individual explants using a binocular light microscope (Stereomaster microscope, Fisher Scientific, U S A). However, global morphogenic responses were pictured using an AT&T GoPhone photo camera. For convenience, only morphogenic responses observed directly from original explants were recorded for statistical analysis. Repetitive organogenic responses following the splitting of responding original explants were not included in the analysis. Shootlets of at least 2.5 cm long were split and transferred onto MS medium without growth regulators for root induction.

Experimental design and statistical analysis

The study used the randomized factorial design. Data were analyzed using linear models with interactions among factors. The analyses were performed with R and R Studio software (version 3.6.0, 2019). The statistical package used for analysis of variance was *emmeans* (Searle et al., 1980) and plotting and graphing were achieved with the software *ggplot2* (Wickham, 2016). The statistical significance of differences among sample means was tested with the Tukey test at 5% level.

RESULTS

The present report pioneers successful shoot organogenesis in daylily bract tissue, demonstrating that it can be reliably applied as a primary explant for *in vitro* propagation. The results of callus, bud and shoot, and root inductions are presented in Figures 1 to 6.

Callus and indirect shoot formation

Within five days of culture, differential cell divisions were observed in cut wound of explants cultured. However, within ten days and thereafter, treated explants developed greater mitotic activities with significant callus formation than negative control explants (Figure 2). Overall, explants cultured on control medium without growth regulators did neither induce organogenesis (shoots or roots) nor callus and subsequently necrotized (Figure 2A). Callus was variably colored, friable, and shoot organogenic (Figure 2B). Indirect shoot

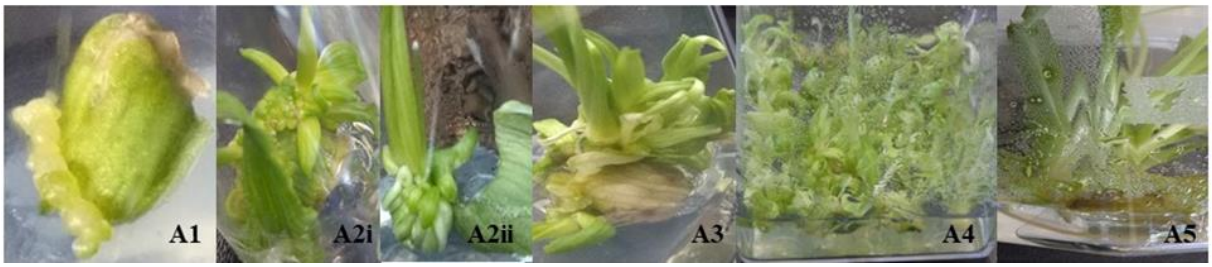

Figure 3. Direct shoot organogenesis and development. A: 21-day-old early multiple shoot bud formation at the bract cut base; A2: 33-day-old mid multiple shoot development, across the wounded area (A2i) or in partial wounded area (A2ii); A3: 45-day-old multiple shoots, ready for splitting to root; A4: 47-day-old freshly split shoots on full-strength basal MS medium for rooting; A5: Rooted shoots ready for greenhouse acclimatization.

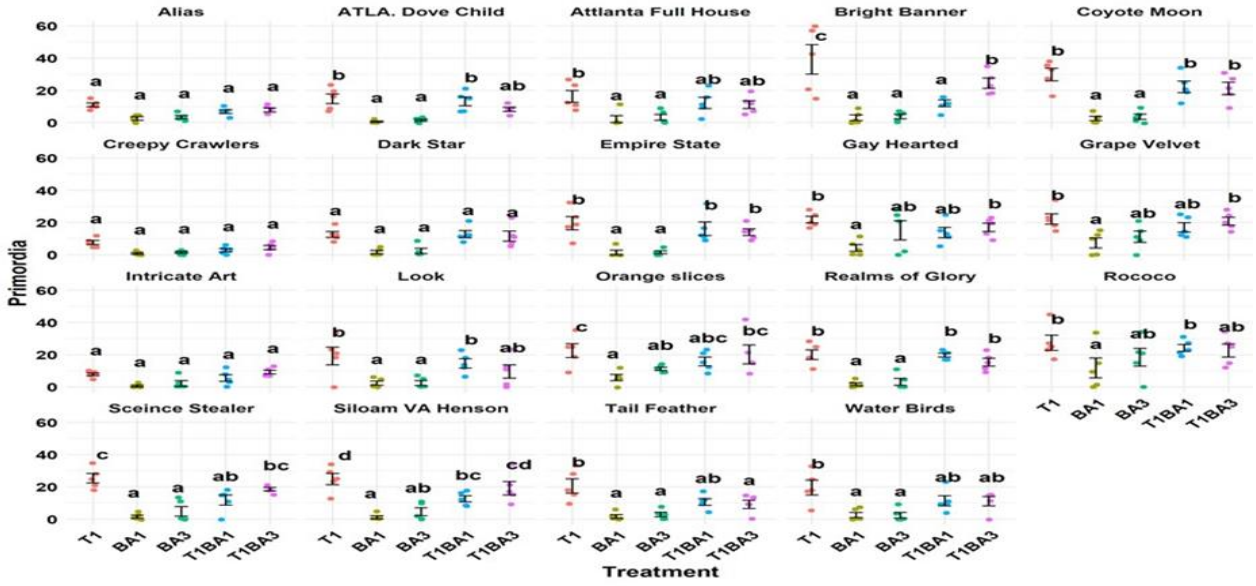

Figure 4. Interactive responses of shoot primordia (Primordia) to growth regulators (T0=0 mg/L TDZ, T1=1mg/L TDZ, BA0=0=mg/L, BAI=1 mg/L BA, and BA3=3 mg/L BA), explant and varieties; five replicates per treatment; bars are mean standard error; and mean differences were tested at 5% level of significance with Tukey test using R and R studio software. Because of the lack of response to T0 and BA0, no related data are presented in Figure 4.

organogenesis occurred within at least 40 days of culture. Callus splitting and sub-culturing speed up multiple shoot formation (Figure 2C) and could potentially extend repetitive multiple shoot induction, indefinitely.

Direct shoot organogenesis

Induction of shoot primordia

Direct shoot organogenesis occurred without callus intervening phase (Figure 3A1) and was more prominent than indirect shoot organogenesis that overall occurred only in less than 5% of explants cultured. Direct shoot formation was typically preceded by the protuberance of a group of cells that bulged in the basal wounded area of the bract explant. Those cellular bulges are termed *shoot primordia* that characteristically developed across (Figure 3 A2i) or partially (Figure 3 A2ii) wound areas where buds and shoots subsequently formed and were an important indicator of the level of shoot organogenesis potential in individual explants. Shoot primordia were observed within two weeks of culture. However, not all primordia developed into buds and shoots, successfully. The amount of induced shoot primordia in individual explants ranged from 0 to 60 and was genotype dependent (Figure 4). The greatest number of shoot primordia (60) was induced in the cultivar ‘Bright Banner’. The average shoot primordia ranged from 0.6 (‘Intricate’) to 39.2 (‘Bright Banner’) per explant. The five top cultivars with

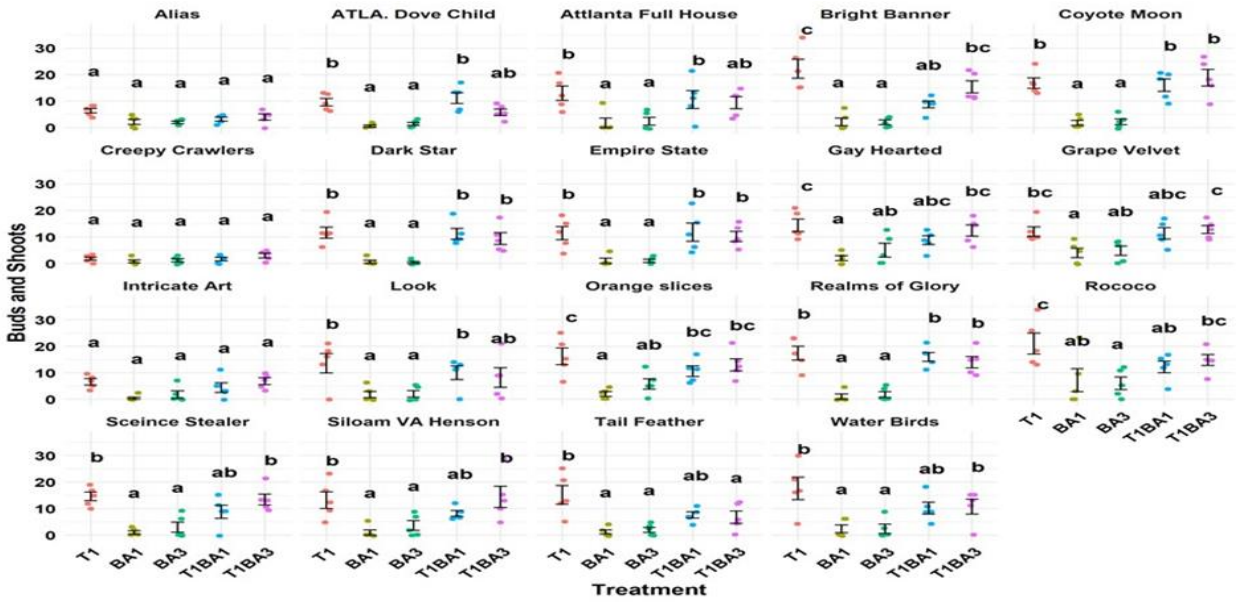

Figure 5. Interactive responses of shoot buds and shoots development to growth regulators (T0=0 mg/L TDZ, T1=1 mg/L TDZ, BA0=0=mg/L, BAI=1 mg/L BA, and BA3=3 mg/L BA), explant and varieties; five replicates per treatment; bars are mean standard error; and mean differences were tested at 5% level of significance with Tukey test using R and R studio software. Because of the lack of response to T0 and BA0, no related data are presented in Figure 5.

greatest average shoot primordia included 'Bright Banner' (39.2), 'Coyote Moon' (29.8), 'Rococo' (27.4), 'Scene Stealer' (25.4), and 'Siloam Virginia Henson' (24.8).

Bud and shoot development

The success of the study was predicated on the potential of the cultivars to induce *in vitro* multiple shoots. The development of buds and shoots from primordia was apparent within three weeks of culture. Accordingly, the proportion of buds and shoots that successfully developed from individual explants is reported in Figure 5. Although statistically variable, the study overall showed that all nineteen cultivars that were studied induced multiple adventitious shoots based on both genotypic and treatment differences. Developmental stages of direct shoot organogenesis are pictured in Figure 3. The number of buds and shoots formed per individual explants ranged from 0 to 34. The greatest amount of buds and shoots was observed in the cultivars 'Bright Banner' and 'Rococo'. The five top performing cultivars in buds and shoots development were 'Bright Banner' and 'Rococo' (34), 'Water Birds' (30), 'Siloam Virginia Henson' (29), and 'Coyote Moon' (27). Ipso facto, the five top cultivars with greatest average buds and shoots per explant were 'Bright Banner' (22.2), 'Coyote Moon' (18.8), 'Water Birds' (17.6), 'Realms of Glory' (17.4), and 'Orange Slices' (16.2). Six cultivars formed at least 20 buds and shoots per explant in at least two treatments, while 17 out of 19 cultivars formed at least 10 buds and shoots per explant in at least two treatments, during the study period.

When considering the influence of growth regulator treatments on shoot organogenesis, TDZ was the most potent chemical stimulus. The greatest buds and shoots numbers per explant of the five top cultivars were influenced by TDZ treatments. Overall, 100% of the cultivars studied responded more effectively to TDZ than BA non-combination treatments. The cultivars 'Alias' and 'Creepy Crawlers' were overall the least respondents across treatments. The ceiling numbers of buds and shoots per explant in these two cultivars were less than ten.

Conversion of buds and shoots from shoot primordia

The proportion of shoot primordia that developed into buds and shoots successfully was estimated using the following formula and data are presented in Figure 6.

Shoot conversion rate (%) = Cultivar no. of buds and shoots/Cultivar no. of shoot primordia × 100

The estimate of successful shoot conversion rate from primordia is consequential in predicting more accurately expected results of current cultivars, when applied under similar cultural conditions. The general conversion rate ranged from 0 to 100%. Accordingly, the five top cultivars with greatest average conversion rates include 'Water Birds' (93.65%), 'Realms of Glory' (91.59%), 'Coyote Moon' (90.12%), 'Dark Star' (90.25%), and 'Atlanta Dove

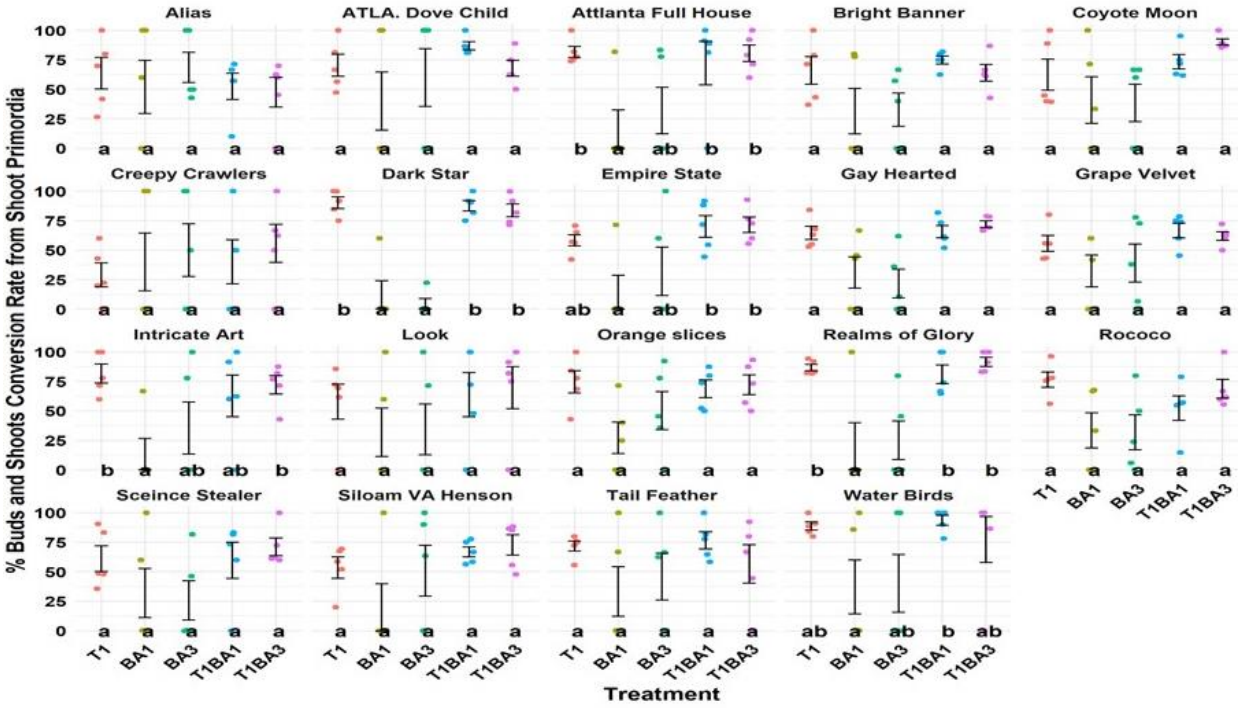

Figure 6. Interactive responses of shoot buds and shoots conversion rates (%) to growth regulators (T0=0 mg/L TDZ, T1=1mg/L TDZ, BA0=0=mg/L, BAI=1 mg/L BA, and BA3=3 mg/L BA), explant and varieties; five replicates per treatment; bars are mean standard error; and mean differences were tested at 5% level of significance with Tukey test using R and R studio software. Because of the lack of response to T0 and BA0, no related data are presented in Figure 6.

Child'. The general response for shoot conversion across cultivars was significantly effective. At least 11 cultivars maxed out the shoot conversion rate in at least three treatments. To highlight the effectiveness of this protocol, each studied cultivar converted buds and shoots form primordia at the minimal rate of 70% in at least three treatments. Based on individual non-combination treatments, the perfect conversion rate was recorded in response to the treatments 1 mg/l TDZ, 1 mg/l BA, and 3 mg/l BA in at least 8, 10, and 9 cultivars, respectively. Similarly, the perfect conversion rate was also observed in 9 and 8 cultivars in response to 1 mg/l TDZ and BA, and 1 mg/l TDZ and 3 mg/l BA combination treatments, respectively.

Root formation and hardening

Separating shootlets that have reached at least 2.5 cm long from the clump (Figures 2C3/3A3 and 3A4) and culturing it on a full-strength MS medium without growth regulators was successful in inducing roots, under the same cultural conditions. More than 95% of shootlets formed roots (Figure 3A5). Lids of the receptacles containing rooted shoots were removed to acclimate the plantlets to the ambient pressure and other environmental conditions, for three days prior to moving it to potted soil, Berger Germinating Mix, BM2 (www.berger.ca), in the greenhouse. All plants grew healthy and normally.

DISCUSSION

This study has successfully pioneered the application of bract as the main explant for micropropagation of daylilies, for all functional purposes. Because callus and shoot formation occurred only at the base cut of the explant, it underpins the concept that stressful wounding releases chemical signals among which are those that induce morphogenesis (Ikeuchi et al., 2013; Chen et al., 2016; Fehér, 2016). Similar observation has previously been reported in globe artichoke bract tissue culture during which shoot organogenesis occurred in the lower region of the bract explants (Ordas et al., 1990). Despite that callus and/or organs may occur naturally in plants or tissue culture without exogenous growth regulators (Chen et al., 2016; Ikeuchi et al., 2019; Zhang et al., 2019), the addition of plant hormones was essential for obtaining positive responses in this study, because the lack of it in the negative control explants' nutrients medium resulted in necrosis without morphogenesis.

It is clearer that either direct or indirect shoot organogenesis can result from applying daylily bract tissue in *in vitro* culture. Remarkably, despite variations

shoot organogenesis occurred across genotypes and was more effective in response to TDZ treatments. This establishes the bract as a reliable explant for daylily *in vitro* propagation. The application of TDZ in previous tissue culture studies has often resulted in very effective responses across plant species and superior performances over different other growth regulators (Huang et al., 2020; Chen et al., 2020). Because of the differential level of difficulty to micro-propagate plant species, there is an exhaustive effort to explore a spectrum of plant tissues, including the bract, for their totipotency potential. Accordingly, the bract tissue has been explored in different plant species for its capacity to induce *in vitro* mass adventitious plants. Although previous results varied, ranging from mere protoplast in banana (Matsumoto et al., 1988) and callus in gladiolus (Bajaj et al., 1983) development to complete organismal development in banana and globe artichoke (Smitha et al., 2017; Comino et al., 2019), the interest in bract tissue culture is growing. The present results are encouraging and show similarities to standard explants that are commonly applied in tissue culture. For example, the use of bract tissue has led to plant development via both direct and indirect shoot organogenesis in different plant species (Ordas et al., 1990; Ninghui et al., 2001; Comino et al., 2019). Other studies have shown that plants can be produced in bract tissue culture via somatic embryogenesis (Divakaran and Nair, 2011; Smitha et al., 2017). Considering that this investigation tested a larger number of genotypes, the results are therefore more extensive. Thus, the contribution of individual explants was better estimated to enable efficient activity planning and predicting of expected results for cost-efficiency, when current genotypes are applied under similar conditions.

Conclusion

The present study has established, for the first time, that daylily bract tissue can be reliably and effectively applied as the principal explant for daylily micropropagation. Considering the extent and diversity of the samples and across the board shoot organogenic success under unrestricted lab environmental conditions, it is anticipated that this protocol will be reproducible more convincingly in broad environmental conditions.

CONFLICT OF INTERESTS

All authors have not declared any conflict of interests.

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