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Full Length Research Paper

A more improved protocol for *in vitro* shoot organogenesis in daylily (*Hemerocallis* sp.)

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Daylily is a crop of landscape, nutritional, and medicinal importance. Despite relative progress, daylily is one of the least explored crops in tissue culture, partly, because of its low responsiveness for *in vitro* organogenesis. This study reports a more efficient one-step protocol for shoot organogenesis. Leaf, flower, flower bud, stem, and root tissues were investigated for shoot organogenesis after different growth regulator treatments on Murashige and Skoog medium. Three out of four of the daylily formed shoots. The variety 'Summer Echoes' was the best of all the other varieties with the greatest average shoots per explant (118) and shooting explant rate 87% when 6-benzyl-aminopurine (BA) and kinetin were used singly. This protocol is the most improved, over the current ones, for shoot organogenesis in daylily. The plant grew normally in the greenhouse.

Key words: Daylily shoots organogenesis, plant micropropagation, daylily tissue culture, *in vitro* plant regeneration.

INTRODUCTION

Daylily is a lucrative horticultural crop, which is increasingly popular, primarily because of its landscape (Dunwell, 2000) and floral beauties (Garber, 2004). It is also used as a food vegetable (Pollard et al., 2004; Knight et al., 2004) and medicinal plant (He, 1994; American *Hemerocallis* Society, 2007). Daylily market is particular, because of the prominence of cultivar names preference. Thus, with unprecedented increasing market demand and development of new varieties with desirable characteristics, vegetative propagation preponderates over seed multiplication to meet the market demand. This is exacerbated by the prominence of seed dormancy among daylily genotypes (Griesbach and Voth, 1957; Apps, 1995) and zygotic embryo poor development in some genotypes, such as, triploids (Li et al., 2008); and cumulatively, it limits seed use mostly to breeders' applications (Dunwell, 2000).

Naturally, a single daylily plant can generate a few new divisions per growing season. At such reproductive lower rate, it can take up to 20 years for an outstanding cultivar to move from enthusiast to mass market (Pounders and Garton, 1996). Efforts towards improving classical *in vivo* techniques for enhanced daylily propagation are encouraging (Leclerc et al., 2006; Leclerc et al., 2005; Dunwell, 2000; Druse, 1997); and the most promising approach has been the application of cytokinin and/or auxin on sheared daylily fans (Amling et al., 2007; Dunwell, 2000; Druse, 1997; Kirby-Smith and Kasha, 1981). This method can generate up to 40 or more new plants per original fan, in a single growing season (Druse, 1997). Although the results are greater than the control, they are inefficient compared to the potential outcomes from *in vitro* tissue culture approach. Further, even if the cultivar forms a relatively large number of divisions per year, it can take 10 years or more to have adequate plants to meet market demand, partly because of nursery production shortage (Dunwell, 2000). Beyond the mere perception, there is globally very limited research on day-

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Abbreviations: BA, 6-Benzyl-aminopurine; **2, 4-D,** 2, 4-dichlorophenoxyacetic acid.

lily micropropagation, primarily because it is less responsive for *in vitro* tissue culture applications. Thus, any efforts emphasizing the development of or enhancing for more efficient and rapid daylily plant multiplication *in vitro*, as highlighted here, is encouraged.

Generally, current methods for micropropagation of daylily apply at least two-step protocols for plant formation (Pounders and Garton, 1996; Heuser and Apps, 1976; Krikorian and Kann, 1981; Meyer, 1976; Krikorian, et al., 1988; Krikorian and Kann, 2002). The initial step of the protocols applies a treatment for callus induction, which generally is distinct from the follow-up growth regulator treatment applied for organ formation. This study applied successfully a single-step protocol treatment for callus and/or shoot formation.

MATERIALS AND METHODS

Daylily genotypes were supplied by the Oklahoma Daylily Association. The plant materials including four genotypes, namely, Grace My Love, Creed Moon, Red Grace, and Summer Echoes were selected from popular cultivars of the State of Oklahoma daylily market.

Explant preparation and cultural conditions

All plant organs including young leaf, stem, root, flower, and flower bud were freshly collected, washed under running tap water for 1 h for cleaning, and surface-sterilized by rinsing with 100% ethanol for 1 min followed by immersing into 25% Clorox for 3 min; the materials were rinsed three times with sterile distilled water. Under continuous sterile conditions of a laminar air-flow hood (Baker Company, Inc.), all tissues were prepared for culture by slicing it into 3 mm long (root, stem, and flower bud) or 3 mm^2 (leaf and flower) size. Excised tissues were cultured on a shoot-inducing medium consisting of Murashige and Skoog (MS) salts and vitamins (1972) and sucrose (30 g/l). All explants were cultured on Petri dishes and wrapped with parafilm. Individual Petri dishes contained five explants; and ten plates were randomly assigned to individual treatments. Each explant was treated as an experimental unit for observations and data collection. This totals the global number of replicates to 50. When callus formed from individual explants, it was sub cultured into several units on a single plate and related shoots were pooled for average per explant. Otherwise, shoots were counted from non- callusing explants for averaging. The final pH of the medium was adjusted to 5.8 with 1 N NaOH after the addition of phytagel (8 g/l) and the media were autoclaved at 121°C for 20 min. MS medium was supplemented with 5, 10, or 15 mg/l 6-benzyl-aminopurine (BA) or kinetin, or 2 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) used alone or in combinations for morphogenesis probe. The basal MS medium was used as control. Cultures were incubated at room temperature. Explants were sub cultured onto fresh media every three weeks. All chemicals were purchased from Sigma Co. (St. Louis, MO).

Observations, data collections and statistical analysis

Observations were made daily on individual plates. Data were collected from individual explants throughout the two-month-experimental period. The standard error was used to assess the level of experiment error.

RESULTS AND DISCUSSION

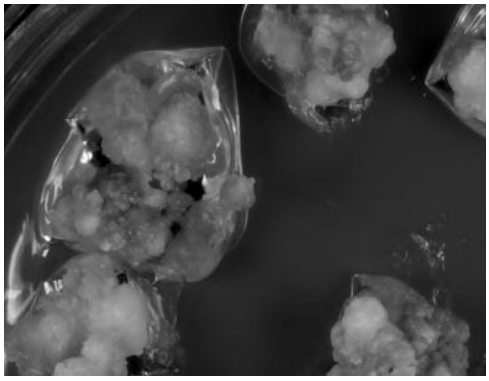
The overall results show variable responses amongst genotypes for both callus and shoot organogenesis (Tables 1 and 2).

Callus formation

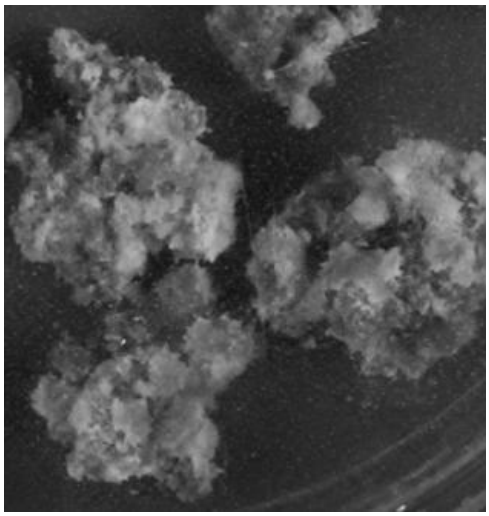
Most explants and explant types evaluated formed callus within a week, except root and stem, whose outcomes were not included in the general results as presented in Tables 1 and 2. The degree of callus formation varied with the genotype and treatment. Prolific callus was generally observed on MS medium containing 2, 4 D and either cytokinins (Table 1). Little callus was observed on the medium containing 2, 4 D alone; and no callus was observed on medium containing cytokinin alone. These results generally agree with a recent study by Li et al. (2010), in which the combination of naphthalene acetic acid (NAA) and BA was required for improving induced callus's quality and organogenic potential. Callus was categorized into non-organogenic and organogenic callus. Organogenic callus was friable, initially whitish and turned green overtime with meristem development, and was observed from explants of cultivars Grace My Love, Creed Moon, and Summer Echoes (Figure 1a). Non-organogenic callus (Figure 1b) was non-friable and unusually totally greenish from initiation throughout its development and somewhat soft and fluffy, but different from snow callus, and was observed from explants of the cultivar Red Grace. Due to the poor quantity and quality, no callus induced by 2, 4 D alone was used for shoot organogenesis.

Shoot organogenesis

A two-week-old callus, which was used to induce adventitious shoots in this study, formed shoots within three weeks in three of the four genotypes that were evaluated, including, 'Grace My Love', 'Creed Moon', and 'Summer Echoes' (Tables 1 and 2). The results show that genotypic difference, for shoot organogenesis, was persistent throughout this study and has also been reported earlier in daylily (Adelberg, 2007; Pounder and Garton, 1996). The genotype Red Grace did not form shoots, primarily, because of its inability to form organogenic callus (Tables 1 and 2). Overall, shoots average per explant and shooting explant rate ranged from 21 to 118 and 33 to 87%, respectively (Table 2). Generally, Summer Echoes responded more efficiently with greatest shooting explant rate (87%) and shoots average per explant (118) (Table 2). Explant shoot formation results were from pooling shoots from calli derived from individual original explants (Figures 2a and b) during the 60-day-study period. Despite that the present study was completed in a relatively shorter period than previously reported studies, the results were greater than those of previously reported pro-



a.

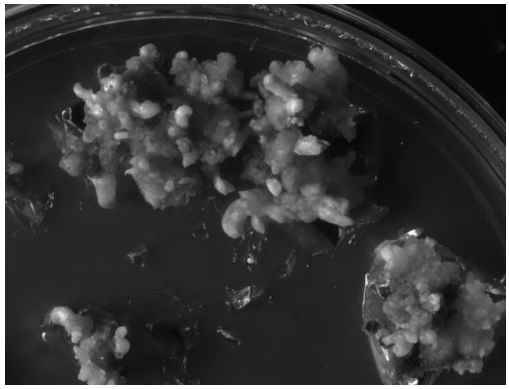


b.

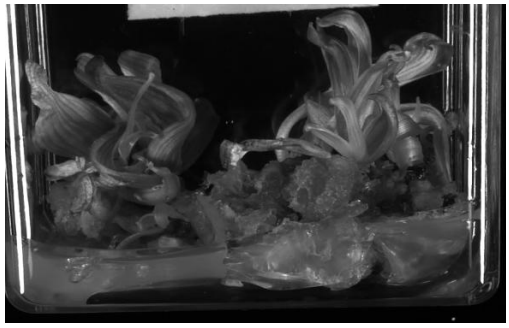
Figure 1. a. Organogenic callus of daylily. **b.** Non-organogenic callus of daylily.

protocols for daylily shoot organogenesis (Meyer, 1976; Heuser and Apps, 1976; Pounders and Garton, 1996; Churikova, 2008; and Li et al., 2010).

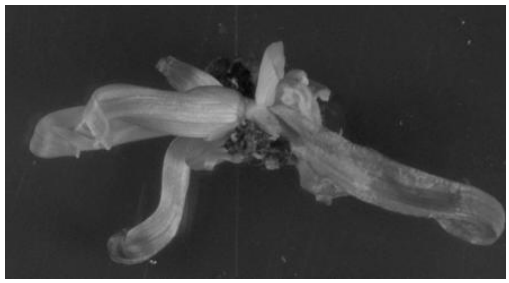
As indicated earlier, only callus cultured on media containing both 2, 4 D and cytokinin was used for shoot organogenesis. The results show that, when callus was subcultured on similar medium for shoot induction, shoot formation occurred in all responsive genotypes. However, when callus was transferred from the initial medium to the medium containing only a cytokinin, shoot formation overall improved (Table 2). This observation also underpins related results that were reported by LI et al. (2010). In addition to the combination treatment of 2, 4 D and BA, the authors observed that the subculturing of callus on the medium containing cytokinin alone increased significantly the development of organogenic callus and shoot regeneration. Noticeably, the present results show that shoot formation improvement was more efficient on media containing cytokinin at greater concentrations (10 and 15 mg/l), compared with that from media containing



a.



b.



c.

Figure 2a. Early callus shoot formation in daylily. **b.** Advanced callus shoot formation in daylily. **c.** Direct shoot formation in flower explants of daylily.

Figure 3. *In vitro* produced daylily plant growing normally in greenhouse.

Table 1. Daylily callus formation.

Treatment	Grace My Love			Creed Moon			Summer Echoes			Red Grace		
	Leaf	Flower bud	Flower	Leaf	Flower bud	Flower	Leaf	Flower bud	Flower	Leaf	Flower bud	Flower
Control	-	-	-	-	-	-	-	-	-	-	-	-
2 mg/l 2, 4-D	+	+	+	+	+	+	-	-	-	+	-	+
2 mg/l 2, 4-D + 5 mg/l Kinetin	+	+++	++	+	+++	++	+	+++	++	+	++	++
2 mg/l 2, 4-D + 10 mg/l Kinetin	+	+++	+++	++	+++	+++	+	+++	+++	+	+++	++
2 mg/l 2, 4-D + 15 mg/l Kinetin	+	++	++	+	++	++	-	++	++	+	++	++
5 mg/l Kinetin	-	-	-	-	-	-	-	-	-	-	-	-
10 mg/l Kinetin	-	-	-	-	-	-	-	-	-	-	-	-
15 mg/l Kinetin	-	-	-	-	-	-	-	-	-	-	-	-
2 mg/l 2, 4-D + 5 mg/l BA	+	++	++	++	++	++	-	+	++	-	++	++
2 mg/l 2, 4-D + 10 mg/l BA	+	++	+++	+++	+++	+++	-	+++	+++	-	++	++
2 mg/l 2, 4-D + 15 mg/l BA	+	++	++	++	+++	++	-	++	+	-	++	++
5 mg/l BA	-	-	-	+	-	-	-	-	-	-	-	-
10 mg/l BA	-	-	-	+	-	-	-	-	-	-	-	-
15 mg/l BA	-	-	-	+	-	-	-	-	-	-	-	-

-, No callus growth; +, little callus growth; ++, median callus growth; +++, large callus growth; root and stem did not form callus.

BA or Kinetin (5 mg/l). These media with greater concentrations of cytokinin caused the most and greatest shoots averages per explant and shooting explant rates (Table 2). 15 mg/l Kinetin-containing medium caused overall better shoot formation. No medium containing 2, 4 D alone or in combination with kinetin 5 mg/l induced shoot formation.

The benefit of cytokinin is not limited to daylily *in vitro* tissue culture. Its application *in vivo* has significantly improved related classical techniques for daylily multiplication (Leclerc et al, 2006; 2007; Dunwell, 2000; Druse, 1997). It has been justified that its application can overcome the inhibitory effects of internally-produced auxin on vegetative bud elongation (Krikorian and Kann, 2002). Repetitive multiple shoots were observed beyond the experimental period. Thus, it was not accounted for in the present data. Occasional direct shoot formation was also observed in flower explants (Figure 2c).

Root formation

Shoots root formation was observed in 92% of shoots within two weeks after shoots have been transferred onto half-strength basal MS medium. All plants grew healthy and normally (Figure 3) in the greenhouse.

Conclusion

This study reports a simple and more efficient protocol for shoot organogenesis in daylily. Under study conditions, three out of four genotypes tested formed shoots. The greatest average shoots per explant and shooting explant rate were 118 and 87%, respectively. Although callus formed in all genotypes, Red Grace callus was not organo-

Table 2. Flower bud callus shoot organogenesis in daylily.

Treatment	Grace My Love		Creed Moon		Summer Echoes		Red Grace	
	Explant Shoot average	Shooting explant rate (%)	Explant shoot average	Shooting explant rate (%)	Explant shoot average	Shooting explant rate (%)	Explant shoot average	Shooting explant rate (%)
Control	0	0	0	0	0	0	0	0
2 mg/l 2, 4-D	0	0	0	0	0	0	0	0
2 mg/l 2, 4-D ± 5 mg/l kinetin	0	0	0	0	0	0	0	0
2 mg/l 2, 4-D ± 10 mg/l kinetin	21±0.8	33±1.1	35±1.2	33±1.2	56±2.2	55±2.0	0	0
2 mg/l 2, 4-D ± 15 mg/l Kinetin	58±1.8	48±1.5	67±2.5	56±2.2	88±4.0	67±2.5	0	0
5 mg/l Kinetin	60 ±2.4	51±2.0	69±3.0	61±2.5	89±4.8	67 ±2.6	0	0
10 mg/l Kinetin	73±3.4	66±2.3	88±4.3	75±3.5	111±5.6	75±3.5	0	0
15 mg/l Kinetin	81±4.2	78±4.0	97±5.1	75±3.6	118±5.2	84±3.8	0	0
2 mg/l 2, 4-D ± 5 mg/l BA	0	0	0	0	0	0	0	0
2 mg/l 2, 4-D ± 10 mg/l BA	47±1.5	38±1.3	51±2.5	56±2.1	57±2.1	57±2.1	0	0
2 mg/l 2, 4-D ± 15 mg/l BA	59±2.3	55±2.1	67±2.5	61±2.1	73±3.3	67±2.6	0	0
5 mg/l BA	58±2.3	57±2.1	65±2.3	63±2.5	75±3.5	70±2.8	0	0
10 mg/l BA	65±2.3	68±2.8	71±3.1	69±3.0	89±4.4	78±3.8	0	0
15 mg/l BA	82±3.8	71±3.3	91±4.5	76±3.7	104±5.3	87±4.8	0	0

Values are mean± standard error.

genic; thus, no shoot formation resulted from it. Genotypic differences were observed for both callus and shoot formation. All plants grew normally in the greenhouse. No callus formed in media containing cytokinin alone as well as in the control medium. Similarly, no shoots formed in control medium or media containing 2, 4-D alone or in combination with kinetin 5 mg/l.

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