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Full Length Research Paper

In Vitro Regeneration of *Rudbeckia hirta* 'Plainview Farm' from Leaf Tissue

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Rudbeckia hirta **'Plainview Farm', a new multiple-layered ray flowered cultivar, shows potential for potted plant production. After years of seed germination, this specific flower morphological trait was still unstable from generation to generation. To maintain its unique features, leaf sections (0.25 cm^2) were cultured on Murashige and Skoog (MS) medium supplemented with either BA (0.5, 1.0, or 2.0 $mg·L^{-1}$), KIN (2.5, 5, or 10 $mg·L^{-1}$), or ZT (0.5, 1.0, or 2.0 $mg·L^{-1}$)toinduce callus and microshoots. After cultivation for 33 days, all cytokinin treatments significantly induced callus and the callus size were 1.5-to-2.4-fold bigger than those withoutcytokinin. KIN at 2.5 $mg·L^{-1}$ was the best treatment for callus induction and microshoot formation. Four microshoots per explant wereproduced at KIN of 2.5 $mg·L^{-1}$. For rooting, all induced microshoots were cultured on MS medium at its one-quarter strength containing either IBA or NAA at 0.5, 1.5, or 3.0 $mg·L^{-1}$. All microshoots formed roots at 0.5 or 1.5 $mg·L^{-1}$ IBA, or 0.5 $mg·L^{-1}$ NAA. There were no significant differences in number of roots per shoot and length of roots among treatments. The plantlets were transplanted, acclimated in a mist system, and grown in a greenhouse. A total of 96.4% of the plants derived from tissue culture had multiple layers of ray flowers, while only 9.6% of the plants from seed propagation did. Therefore, in vitro regeneration of *R. hirta* 'Plainview Farm' was a feasible way to rapidly produce uniform plants with multiple layers of ray flowers.**

Keywords: Blackeyed Susan, Callus, Microshoot, Somaclonal variation

INTRODUCTION

Rudbeckia hirta L. (Blackeyed Susan), a member of Asteraceae, is a perennial plant native in North America. Blackeyed Susan has long been used in the traditional medicine of American indigenous people as a remedy against infections (Constabel et al., 1988). This species has been recently demonstrated to be an antagonistic plant to nematodes (Daimon and Mii, 1995). Blackeyed Susan is a popular garden flower and landscape plant.

Many cultivars have been bred for ornamental purposes such as 'Bambi', 'Double Gloriosa', 'Gloriosa', 'Irish Eyes', 'Supurba', 'Toto' (Floridata.com LC., 2011), 'Plainview Farm', and 'UMaine'(Zhang, 2001). *R. hirta* 'Plainview Farm', selected from an open-pollinated population, has multiple layers of ray flowers (Zhang, 2001). This cultivar shows the potential for potted plant production and landscape. Similar to other *Rudbeckia* species and cultivars, this cultivar is propagated by seeds. The authors have observed seed propagation of *R. hirta* 'Plainview Farm' over years, but the unique morphological trait of multiple layers of ray flowers was unstable (unpublished data).

The selection and production of new varieties require both traditional plant breeding methods and biotechnological means. Tissue culture is an efficient tool for mass propagation of *Rudbeckia* species. Protoplasts

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isolated from leaves, cotyledons, ray florets, and callus culture of seedlings of *R. hirta*, *R. laciniata* L., and *R. purpurea* L. regenerated shoot buds on regeneration medium (Al-Atabee and Power, 1987). Somatic hybrids of *R. hirta*'Marmalade' and *R. laciniata*'Irish Eyes' were regenerated via rhizogenesis on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.1 mg·L^{-1}3-indoleacetic acid (IAA), and 0.4 mg·L^{-1}6-furfurylaminopurine (kinetin, KIN) (Al-Atabee et al., 1990). Cotyledon sections of *R. hirta* were initiated to induce callus on Schenk and Hildebrandt basal medium (Schenk and Hildebrandt, 1972) supplemented with 5 mg·L^{-1}2.4-dichlorophenoxyacetic acid (2,4-D), and 5 mg·L^{-1}KIN (Luczkiewicz and Cisowski, 2001). Daimon and Mii (1995) reported that adventitious shoots were induced on the surface of seedling-derived hairy roots of *R. hirta* after 30-50 days of culture on half strength MS agar medium supplemented with 0.5 or 1.0 or mg·L^{-1} 6-benzylaminopurine (BA) and 0.1 mg·L^{-1} α-naphthaleneacetic acid (NAA). Plants regenerated from hairy roots had morphological alterations such as wrinkled leaves, small size in flowers, and abundant lateral branching of roots.Szarvas et al. (2006) developed a micropropagation protocol using seedlings' meristems of *R.hirta*. Shoot-buds developed from seedling axillary meristem on half-strength MS medium containing 10 mg·L^{-1} KIN, or 2 mg·L^{-1}KIN plus 0.1 mg·L^{-1}N6-(2-isopentenyl)-adenine (2iP) and elongated roots formed on MS medium with 2 mg·L^{-1}IAA. However, a drawback of regeneration from seeding is that propagation of a specific cultivar is impossible due to seedling variability. Therefore, leaf tissue as a source of explants appears to be an ideal choice for producing uniform plants with unique flower features for nursery growers. A large number of plants, which are true to type, can be generated.

The purposes of this work are to

1) develop in vitro regeneration techniques of *R. hirta* 'Plainview Farm' using leaf tissue, and

2)determine how efficiently the regeneration techniques maintain the unique flower feature.

MATERIALS AND METHODS

Induction of callus and microshoots

A 7.8-L potted plant of *R. hirta* 'Plainview Farm' was grown in a greenhouse for two months and used as a source of explants. New leaves were harvested, wrapped with wet paper towels, and brought into the lab. The leaves were soaked in 70% ethanol for 10 seconds, then disinfested using 10% ultra-bleach (6.0% sodium hypochlorite; Wal-Mart Stores, Inc. Bentonville, AR) supplemented with 7 drops of Tween 20 (ACC00528/0030, Agdia® Inc., Elkhart, IN) for 10 minutes. All leaf tissues were rinsed three times using sterile distilled water. The leaf sections (0.25 cm^2) were dissected and loaded into 60-mL Pyrex glass tubes containing 10 mL MS basic medium plus 30 g·L^{-1} sucrose, 8 g·L^{-1} agar (Sigma Chemical Co., St. Louis, MO) and different kinds of cytokinins for callus and shoot induction. The pH of the media was adjusted to 5.8 with NaOH or HCl before adding agar. A total of 10 mL of the mediawas pipetted into glass tubes which were covered with caps and autoclaved at 121^{o}C for 30 minutes. All tubes with explants loaded into tube racks and placed in plastic bags. The treatments were arranged randomly on the shelves in agrowth room. All cultures were incubated in thegrowth room at a temperature of 27.2 ± 1.9 ^{o}Cwith a 16-h photoperiod (138 μmol·m^{-2}·s^{-1} photosynthetic photon fluxfrom cool-white fluorescent lamps).

BA,6-(4-hydroxy-3-methyl-trans-2-butenyl-amino) purine (zeatin, ZT) at doses of 0.5, 1, or 2 mg·L^{-1}, and KIN (all three obtained from Sigma Chemical Co., St. Louis, MO) at doses of 2.5, 5, or 10mg·L^{-1} were compared. A total of 36 glass tubes (replications) with one explant per tube were used for each treatment. After 33 days, the diameter of callus and total number of microshoots were recorded. Callus size was calculated using the following equation: [π * (diameter/2)^3 * 4/3].

Root Formation

Microshoots (~1.5 cm) were subcultured on MS media containing 2.5 mg·L^{-1}KIN to increase the number of microshoots. Microshoots (~2.0 cm) were separated and cultured on ¼ strength MS medium plus 30 g·L^{-1} sucrose with either of 0.5, 1.5, or 3.0 mg·L^{-1}3-indolebutyric acid (IBA) or NAA (Sigma Chemical Co., St. Louis, MO) for rooting. A total of 36 glass tubes (replications) with one shoot per tube were used for each treatment. All cultures were maintained at the same conditions as above for 43 days. Number of shoots with roots, total number of roots, the length of the longest root (cm), total number of shoots, and the height of the tallest shoots (cm) were recorded.

Acclimatization and transplanting

All plantlets obtained in vitro were carefully washed using running water to remove agar medium. They were transferred to a tray with 32 cells (5.0 × 5.0 × 6.0 cm^3 cell) contained with a mixture of perlite (Whittemore Company Inc., Lawrence, MA) and PRO-MIX (65-75%, by volume, of Canadian sphagnum peat moss; 35-25% of perlite, vermiculite, macronutrients and micronutrients, dolomitic, and calcitic limestone; Scotts Sierra Horticulture Products Co., Marysville, OH) (3:1 v/v). Flats were placed under intermittent mist. Misting frequency was controlled by a timer (Phytotronics Inc., Earth City MO) set at 20 seconds every 10 minutes for the first 2 weeks, and then reduced to 20 seconds every 20 minutes for the remainder of the experiment. By lengthening the interval between misting, the relative humidity (RH) was gradually decreased from 100% to 70%. No additional light was provided. After 25 days of acclimatization, regenerated plants were transplanted into 6 inch standard thinwall round pots with Metro-Mix 560 Scotts Coir growing medium (Scotts Sierra Horticulture Products Co., Marysville, OH). Another set of seed germinated plants was also grown in the same greenhouse. Plants were randomly arranged on greenhouse benches with three replications and 28 plants per replicate. When blooming, the number of single, double or multiple layers of ray flowers was counted.

Analysis of variance (ANOVA) was performed using JMP v 9.0 (Statistical Analysis System, Cary, NC). The results presented are the means of 36 individual plants with the standard error. Comparisons of means were conducted using Tukey's HSD test. All analyses were regarded as significant at $p < 0.05$.

RESULTS AND DISCUSSION

Induction of callus and microshoots

Cells on the medium-faced leaf tissues began to de-

Table 1: Induction of callus and microshoots of *Rudbeckia hirta* 'Plainview Farm' in response to different cytokinins.

Cytokinin[z]	Concentration ($mg \cdot L^{-1}$)	Callus size (cm^3)[y]	# Microshoots
Control	--	0.55 ± 0.10b[x]	0.0 ± 0.0 d
BA	0.5	1.88 ± 0.33 ab	0.7 ± 0.2 cd
	1.0	1.85 ± 0.27 ab	1.5 ± 0.3 bc
	2.0	3.64 ± 1.51 a	1.7 ± 0.3 bc
KIN	2.5	3.46 ± 0.47 a	4.0 ± 0.4 a
	5	2.13 ± 0.25 ab	2.2 ± 0.4 b
	10	2.27 ± 0.40 ab	2.4 ± 0.4 b
ZT	0.5	1.94 ± 0.30 ab	1.3 ± 0.3 bc
	1.0	1.88 ± 0.29 ab	1.4 ± 0.2 bc
	2.0	1.68 ± 0.28 ab	0.9 ± 0.2 cd
p value		0.01	<0.0001

[z]BA:6-benzylaminopurine, KIN:6-furfurylaminopurine, ZT: 6-(4-hydroxy-3-methyl-trans-2-butenyl-amino) purine.
[y]Mean and standard error of 36 replications are presented.
[x]Different letters in the column indicate that they are significantly different at $p < 0.05$ according to Tukey's HSD mean separation.

Figure 1: *Rudbeckia hirta* 'Plainview Farm' microshootsregenerated on the medium containing 2.5 $mg \cdot L^{-1}$ kinetin.

differentiate and upwards-curled leaf tissue appeared in the first week of culture. Chlorophyll broke down and leaf tissue gradually turned light green. One week later, 33% of leaf sections on the MS media without cytokinins began to turn brownish, while only 6-22% on the media with BA, KIN or ZT (data not shown). These results are not surprising since cytokinins can delay leaf senescence (Taiz and Zeiger, 2006). In the 3rd week, callus was induced on 11-50% of leaf sections on the media with cytokinins, while little callus was observed on the media without cytokinin. Compared to the control without cytokinin, all treatments significantly induced callus ($p<0.0001$). Their callus size after 33 days of culture was 1.5-2.4 folds bigger than that of no cytokinin (Table 1). KIN at 2.5 $mg \cdot L^{-1}$ was the best treatment for callus induction and microshoot formation (Table 1). As previously reported, KIN was successfully used for inducing callus and microshoot formation of *Rudbeckia sp.* (Luczkiewicz and Cisowski, 2001; Luczkiewicz et al. 2002; Szarvas et al., 2006). A total of 4 microshoots per explant were produced at KIN of 2.5 $mg \cdot L^{-1}$ (Table 1 and Figure 1). KIN at 5 and 10 $mg \cdot L^{-1}$ induced about 2 microshoots per explant. However, the average number of microshoots on other media was less than 2. All induced microshoots were subcultured on the MS medium with 2.5 $mg \cdot L^{-1}$ KIN, and the multiplication rate varied from 4 to 10 (data not shown). Szarvas et al. (2006) found that the number of shoots produced on MS media ranged from 3 to 16, depending on the concentration of KIN or KIN plus 2iP.

Root formation

Rudbeckia hirta 'Plainview Farm' is easy to root (Table 2 and Figure 2). After being cultivated on the rooting media for 10 days, 80% shoots rooted on the media without auxin, while only 30-60% and 40-60% rooted on the media with IBA and NAA, respectively (data not shown). All the shoots on media containing no auxin, IBA at 0.5 or 1.5 $mg \cdot L^{-1}$, or NAA at 0.5 $mg \cdot L^{-1}$ were rooted after culture for 43 days. About 90% of shoots rooted on the media with IBA at 3.0 $mg \cdot L^{-1}$ or NAA at 1.5 $mg \cdot L^{-1}$, however, only 72% of shoots rooted for media with 3.0 NAA at $mg \cdot L^{-1}$ (Table 2). It is interesting that rooting percentage decreased as auxin concentrations increased (IBA: $p = 0.0004$; NAA: $p < 0.0001$). This implied that the auxin had inhibited rooting (Taiz and Zeiger, 2006). In particular, NAA had more inhibitory effects than IBA. This result was also observed by Szarvas et al (2006).

Although number of roots, length of the longest roots, number of shoots, and height of the longest shoots varied among treatments, no significant difference was observed in comparison with control (no auxin) (Table 2). The number of roots varied from 4 to 6, while shoot number was 2 to 4. The length of the longest roots ranged from 4.4 cm to 6.0 cm, while height of the longest shoots was 7.8 cm to 9.3 cm (Table 2).

Table 2: Root formation and microshoots growth of *Rudbeckia hirta*‘ Plainview Farm’ in response to different auxins.

Auxin[z]	Concentration ($mg \cdot L^{-1}$)	% Shoots with root[y]	# Roots	Length of roots (cm)	# Microshoots	Length of microshoots (cm)
Control	--	95 ± 2.2 ab[x]	3.8 ± 0.6	4.7 ± 0.4	2.6 ± 0.4	9.1 ± 0.8
IBA	0.5	100 ± 0.0 a	4.1 ± 0.7	5.5 ± 0.5	2.8 ± 0.5	8.3 ± 0.8
	1.5	100 ± 0.0 a	5.8 ± 0.9	5.9 ± 0.5	3.1 ± 0.4	9.3 ± 0.5
	3.0	92 ± 2.5 ab	4.1 ± 0.8	4.4 ± 0.5	2.0 ± 0.3	8.0 ± 0.8
NAA	0.5	100 ± 0.0 a	5.9 ± 0.6	5.7 ± 0.2	3.5 ± 0.6	8.7 ± 0.8
	1.5	88 ± 3.3 b	5.3 ± 0.9	4.8 ± 0.7	2.8 ± 0.4	8.2 ± 0.5
	3.0	72 ± 3.3 c	4.3 ± 0.5	5.2 ± 0.2	2.6 ± 0.2	7.8 ± 1.1
p value		<0.0001	0.2	0.2	0.3	0.8

[z]IBA:3-indolebutyric acid, NAA:α-naphthaleneacetic acid.
[y] Mean and standard error of 36 replications are presented.
[x] Different letters in the column indicate that they are significantly different at $p < 0.05$ according to Tukey’s HSD mean separation.

(Figure B): acclimatized under a mist system

Figure 3 : In vitro regenerated plantlets of *Rudbeckia hirta* ‘Plainview Farm’ acclimatized under the mist system.

Figure 4: In vitro regenerated plants of *Rudbeckia hirta* ‘Plainview Farm’ grew in the greenhouse.

Acclimatization and transplanting

The plantlets were acclimatized in the mist system (Figure 3) and grown in the greenhouse and nursery (Figure 4 and 5). When blooming, the number of each type of flower was counted. A total of 96.4% of the potted plants with multiple layers of ray flowers derived from tissue culture (Table 3 and Figure5), and 3.6% of potted plants from tissue culture have two or three layers of ray flowers. This may result from somaclonal variation, which is common among regenerated plants via callus from tissue culture (Chatterjee and Gupta, 1997; El-Dougdoug et al., 2007; Evans and Sharp, 1986; Roylance et al., 1994). However, only 9.6% of the plants with multiple layers of ray flowers came from seed propagation. A majority of flowers from seed propagation was one to three layers of ray florets (Table 3).

In summary, in vitro regeneration of *R.hirta* ‘Plainview Farm’ from leaf tissue was a feasible way to produce multiple-layered ray flowered plants. Callus and microshoots were induced from leaf tissue on the MS medium supplemented with 2.5 $mg \cdot L^{-1}$ KIN. This was a feasible way to rapidly produce uniform plants with multiple layers of ray flowers.

ABBREVIATIONS

BA: 6-benzylaminopurine;
IBA: 3-indolebutyric acid;
KIN: 6-furfurylaminopurine;
NAA: α-naphthaleneacetic acid;
ZT: 6-(4-hydroxy-3-methyl-trans-2 butenyl-amino) purine

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Table 3: Frequency of flower layers of *Rudbeckia hirta*'Plainview Farm' propagated from seed germination and tissue culture.

Flower layers	Percentage of plants[z]		t-test *p* value
	Seed propagation	Tissue culture	
1	17.9 ± 2.1	0.0 ± 0.0	0.001
2	33.3 ± 1.2	2.4 ± 1.2	<0.0001
3	23.8 ± 1.2	1.2 ± 1.2	0.0002
4	15.5 ± 3.1	0.0 ± 0.0	0.008
5	9.5 ± 3.1	96.4 ± 2.1	<0.0001

[z]Mean and standard error of three replications are presented.

Figure 5: In vitro regenerated plants of *Rudbeckia hirta* 'Plainview Farm'grew and flowered in nursery.

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