

GELLING AGENTS, SILVER NITRATE, AND SEQUESTRENE IRON INFLUENCE ADVENTITIOUS SHOOT AND CALLUS FORMATION FROM *RUBUS* LEAVES*

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(Received 30 January 2001; accepted 3 August 2001; editor S. S. Korban)

SUMMARY

Blackberry (*Rubus* spp.) cultivars Chester Thornless, Kotata, Marion, and Navaho were evaluated for morphogenic potential using the top one to four leaves from *in vitro*-grown shoots. In each experiment adventitious shoots were regenerated via organogenesis without an intervening callus phase. Rooting was spontaneous when shoots were transferred to a medium without growth regulators or with indole-3-butyric acid (IBA). Three gelling treatments (agar, Gelrite, and a combination) calibrated for equal gel firmness [$110 \text{ g}/(1.1 \text{ cm})^2 \pi$] did not affect explant regeneration or the number of shoots per explant, but did affect callus production. Significantly more callus ($P \leq 0.05$) was induced on regeneration medium (RM) with $10 \mu\text{M}$ N⁶-benzyladenine (BA), $0.5 \mu\text{M}$ IBA, and 0.19% Gelrite, than on medium containing either 0.71% agar, or the 0.35% agar/0.11% Gelrite combination. Explants on RM with $5 \mu\text{M}$ IBA produced significantly more callus, but fewer shoots, compared to zero or $0.5 \mu\text{M}$ IBA treatments for all gel treatments. Adding 200 mg l^{-1} sequestrene iron [sodium ferric ethylene diamine di-(*O*-hydroxyphenylacetate)] at the first transfer onto RM induced more shoots per explant than the control, but did not influence the amount of callus produced. Sequestrene iron in the second transfer on RM significantly increased the regeneration (caulogenesis) frequency from 30 to 40% for 'Marion' and from 23 to 43% for 'Kotata'. Silver nitrate significantly reduced callus production for all treatments, but did not affect the frequency of caulogenesis or the number of shoots per explant.

Key words: blackberry; adventitious shoots; gelling agent.

INTRODUCTION

Regeneration of adventitious shoots from leaves, petioles, internodes, cotyledons, and embryos of *Rubus* is an important step in improving cultivars through genetic transformation. Many factors must be considered when optimizing a regeneration protocol. Swartz et al. (1990) enhanced organogenesis of *in vitro* leaf explants from two *Rubus* hybrids using a thidiazuron (TDZ) pretreatment. Silver nitrate or sequestrene iron aid in shoot regeneration for some crops (Taylor et al., 1994; Hyde and Phillips, 1996). Gelling agents may affect the success of micropropagation of apples, pears, and 'Autumn Bliss' and 'Canby' red raspberries (Zimmerman et al., 1995; Chevreau et al., 1997); however agar or gellan gum have no effects on regeneration of other raspberries (Cousineau and Donnelly, 1991). Our objectives include determining the effects of silver nitrate, sequestrene iron, and gelling agents on regeneration of shoots from leaves of several commercially important blackberry cultivars.

*Part of a thesis submitted by C. W. V. Tsao in partial fulfillment of the requirements for the MS degree. The use of trade names in this publication does not imply endorsement by the US Department of Agriculture or Oregon State University.

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MATERIALS AND METHODS

Plant material. *Rubus* spp. hybrid blackberry cultivars Chester Thornless, Kotata, Marion, and Navaho were multiplied *in vitro* on NCCR-RUB medium (Reed, 1990). The medium included MS salts (Murashige and Skoog, 1962) and MS vitamins with double MS iron, $0.29 \mu\text{M}$ gibberellic acid A₃ (GA₃), $0.49 \mu\text{M}$ indole-3-butyric acid (IBA), $4.45 \mu\text{M}$ N⁶-benzyladenine (BA), 0.35% agar (Difco granulated agar, Chicago, IL), and 0.145% Gelrite (Schweizerhall, Piscataway, NJ). The pH was adjusted to 5.7 before autoclaving. Growth-room conditions were 25°C with a 16-h photoperiod (cool white fluorescent illumination, $25 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Shoots were proliferated and maintained in GA7 Magenta boxes (Magenta Corp., Chicago, IL) with a 3–4-wk transfer cycle.

Regeneration procedure. Microshoots (≈ 10 mm long) with the upper four to six leaves were pretreated for 21 d on NCCR-RUB medium containing $1 \mu\text{M}$ TDZ (Swartz et al., 1990). Growth conditions were 20°C with a 16-h photoperiod (cool white fluorescent illumination) in a growth chamber (T173, Hoffman, Albany, OR). Light intensity varied from 15 to $40 \mu\text{mol m}^{-2} \text{ s}^{-1}$ from the front to the back of the chamber. For caulogenesis, leaf explants (top four leaves) from pretreated shoots were placed on RM (NCCR-RUB medium with $0.29 \mu\text{M}$ GA₃ + growth regulators) for 1 wk in the dark, followed by 3 wk in the growth chamber at 20°C with a 16-h photoperiod (RM/T1), and then subcultured on fresh RM (RM/T2) for an additional 3 wk.

Effect of genotype and gelling agents on caulogenesis. Blackberry cvs. Kotata, Marion, and Navaho were evaluated on regeneration medium (RM) with zero or $0.5 \mu\text{M}$ IBA and either 5 or $10 \mu\text{M}$ BA, and gelled with either 0.71% agar, 0.19% Gelrite, or a combination of 0.35% agar and 0.11% Gelrite. BA concentrations (5 and $10 \mu\text{M}$) were chosen based on preliminary experiments. Percent caulogenesis, shoots per explant, and callus index were recorded at the end of the second subculture (7 wk). Callus index was

reported as the amount of callus on the leaf surface using the following classification: no callus (0), 1–20% (1), 21–40% (2), 41–60% (3), 61–80% (4), and 81–100% (5). The percentage of caulogenesis = number of explants producing shoots/number of explants tested (30 or 36 pieces) × 100. Three replicate experiments were analyzed. All data analyses were by the general linear model (GLM) procedure using SAS programming (SAS, 1993). The results of statistical analysis were considered significant at $P \leq 0.05$.

The NCGR-RUB medium containing 0.29 μM GA₃, 0.49 μM IBA, and 4.45 μM BA was used to calibrate gel firmness. A fruit firmness tester (Ametek LKg-1, Hatfield, PA) was used to quantify gel firmness. Difco granulated agar [Fa (firmness) = $-217.09 + 460.49 \times \text{agar concentration} (\%)$], Schweizerhall Gelrite [Fg = $-222.25 + 1792.88 \times \text{Gelrite concentration} (\%)$], and the combination of agar and Gelrite [F_{(0.35%a),g} = $-88.69 + 1792.13 \times \text{Gelrite concentration} (\%)$] were used. A standard firmness for each medium was set as 110 g/(1.1 cm)² π to calculate the standard concentration (%) using a linear regression analysis to provide equal firmness for testing the effect of gelling agents and the additives silver nitrate or sequestrene iron on blackberry regeneration.

Effect of silver nitrate or sequestrene on caulogenesis. Silver nitrate (10 mg l⁻¹) or sequestrene iron [sodium ferric ethylene diamine di-(*O*-hydroxyphenylacetate)] (200 mg l⁻¹) were each tested separately in the first two transfers on RM containing 10 μM BA and either zero or 0.5 μM IBA. The compounds were added either in both RM transfers (+, +), neither transfer (-, -), the first transfer (T1) only (+, -), or the second transfer (T2) only (-, +). The two compounds were not tested together, and neither was included in the pretreatment medium. Explant preparation was the same as in the gelling agent experiment. 'Chester Thornless', 'Kotata', 'Marion', and 'Navaho' blackberries were evaluated for caulogenic response on RM with silver nitrate, and 'Marion' and 'Kotata' were evaluated for caulogenesis on RM with sequestrene iron. Data were taken from three replicate experiments. Contrast analysis was conducted after 49 d on RM on six combinations of silver nitrate or sequestrene iron treatments alone and on their interaction with IBA.

RESULTS AND DISCUSSION

Effect of gelling agents on caulogenesis. Percent caulogenesis and shoots per explant were not significantly different ($P \leq 0.001$) among the three gelling agents (Table 1). Explants on RM with 10 μM BA and no IBA produced the highest percentage of regeneration and the most shoots per explant, but results were not statistically different from most of the other treatments. The gelling agent × growth regulator treatment interaction was significant for caulogenesis ($P \leq 0.01$) and callus formation ($P \leq 0.001$). Multiple shoots without callus were produced from the leaf edge and adaxial surface of 'Marion' and 'Kotata' on Gelrite RM with 10 μM BA and no IBA, one of the best shoot-promoting treatments overall for the three cultivars (Fig. 1A, B). Shoots were produced on 'Thornless Evergreen' on RM with 5 μM BA (Fig. 1C). Callus

induction from leaves on all three gelling agent treatments was significantly enhanced ($P \leq 0.001$) by 0.5 μM IBA (Fig. 1D), so IBA should be avoided if direct organogenesis is desired. Cultivar, gelling agent, and treatment interactions were all significant for callus formation ($P \leq 0.001$).

Researchers commonly use 0.7–0.8% agar or 0.2–0.25% Gelrite to solidify a tissue culture medium (Anderson, 1980; Reed, 1990). Our data confirmed that the 0.7% agar and 0.2% Gelrite have similar firmness in MS medium, and 0.8% agar and 0.25% Gelrite are also nearly equivalent (data not shown). Cousineau and Donnelly (1991) found that increasing agar concentrations from 0.2 to 1.0% have no significant effect on raspberry regeneration. Significantly more callus ($P \leq 0.05$) was induced on RM with 10 μM BA, 0.5 μM IBA, and Gelrite, than on agar or the agar/Gelrite combination (Table 1). The gel type did not significantly influence caulogenesis or shoots per explant. The most shoots per explant and the highest caulogenesis frequency were observed on explants grown on Gelrite RM with 10 μM BA, but this was not significantly better than many of the other treatments used.

Effect of sequestrene iron on caulogenesis. Significantly more explants ($P \leq 0.05$) produced shoots when sequestrene iron was in RM/T2 (-, +) compared to those on RM/T1 when IBA was not included in the medium (Table 2). There were no significant differences in caulogenesis among the treatments when IBA was included in the RM. Sequestrene iron in the IBA 0, RM/T1 (+, -) reduced the number of shoots per explant compared to the control (-, -) but the percent caulogenesis and the callus index were not affected. The percent caulogenesis of 'Kotata' increased from 23% in the sequestrene-free control to 43% with sequestrene iron in the RM/T2 only and 'Marion' increased from 30 to 40% (data not shown). Sequestrene significantly increased the apparent percent caulogenesis of these *Rubus* cultivars when included in RM/T2 (bud enlargement/shoot elongation stage), but not in RM/T1 (bud induction stage). Sequestrene might promote further development of previously induced buds. The response of control 'Kotata' and 'Marion' blackberries were significantly differently (23% and 30%) on standard RM, but their caulogenic responses on RM/T2 with sequestrene were very similar (43% and 40%). This suggested that sequestrene had the potential to promote organogenesis from leaves in other *Rubus* cultivars as well, but an initial screen of nine cultivars indicated that the response was cultivar-dependent (data not shown).

TABLE

CAULOGENESIS FROM WHOLE LEAVES OF 'KOTATA', 'MARION', AND 'NAVAHO' BLACKBERRIES ON MEDIUM SOLIDIFIED WITH THREE GELLING AGENT TREATMENTS

Treatment ^c	Caulogenesis (%) ^a			No. shoots per explant			Callus index		
	Agar	Gelrite	Agar + Gelrite	Agar	Gelrite	Agar + Gelrite	Agar	Gelrite	Agar + Gelrite
IBA 0 + BA 5	10.00 z	0.0 y	13.33 z	4.39 z	0.0 w	3.42 xy	2.29 vw	1.56 u	2.56 w
IBA 0 + BA 10	12.22 z	14.44 z	14.44 z	2.95 wxyz	4.19 yz	3.69 xy	2.54 w	1.93 v	1.77 uv
IBA 0.5 + BA 5	8.89 yz	11.11 z	6.67 yz	2.13 wxy	2.44 wxy	1.33 wxy	3.63 y	4.01 yz	3.80 xyz
IBA 0.5 + BA 10	5.56 yz	10.00 z	0.0 y	0.74 wx	3.48 xyz	0.0 w	3.59 y	4.11 z	3.54 x

n = 90 whole-leaf explants, 30 for each cultivar.

^a Means with the same letter within a group (caulogenesis, no. shoots per explant, callus index) are not significantly different at $P \leq 0.05$.

^b Callus index ranged from 0 (no callus) to 5 (81–100% callus on the leaf surface).

^c NCGR-RUB medium with 2.9 μM GA₃ and concentrations (μM) of BA and IBA as listed.

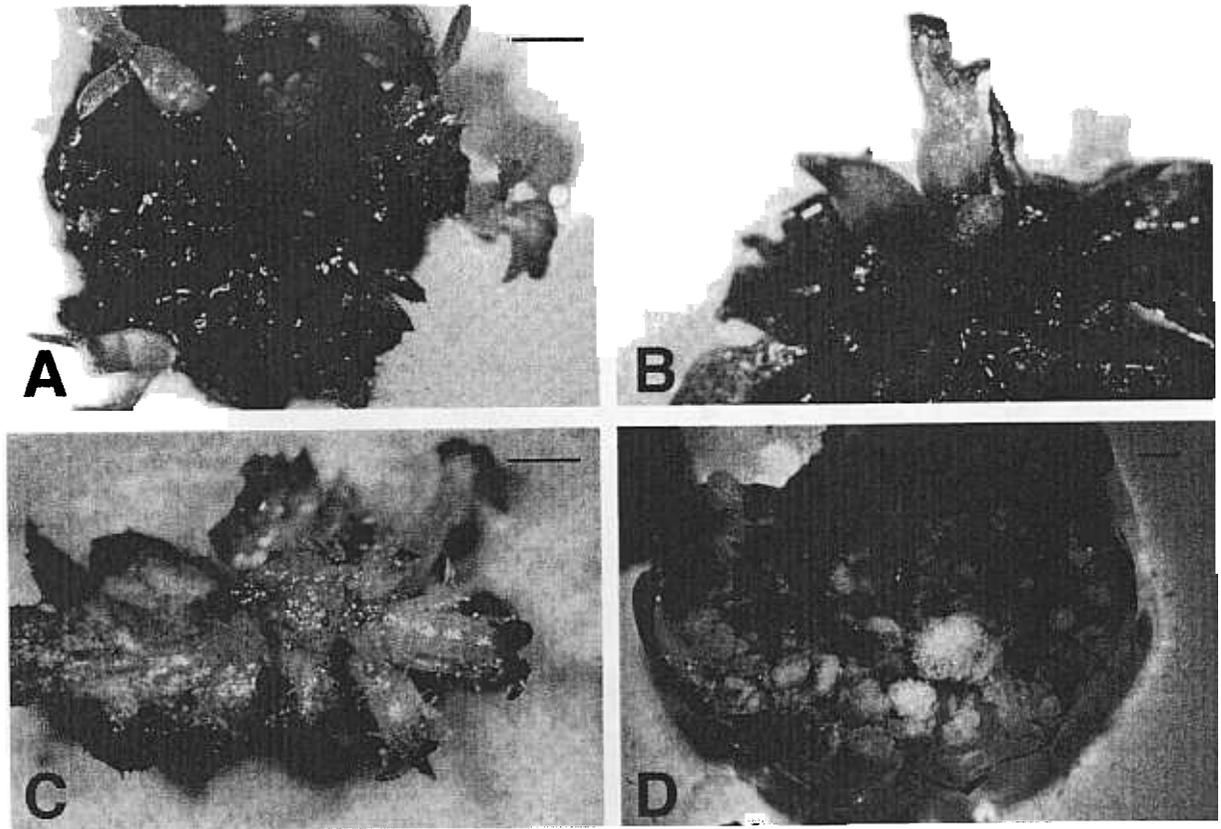


FIG. 1. *In vitro* shoot regeneration from blackberry whole-leaf explants. A, The upper surface of 'Marion' produced multiple shoots after 30 d on regeneration medium with 10 μ M BA (bar = 1.2 cm). B, 'Kotata' produced multiple adventitious buds from the top of the leaf-petiole junction after 21 d on regeneration medium with 10 μ M BA (bar = 2 cm). C, 'Thornless Evergreen' produced multiple shoots from the lower surface of the leaf-petiole junction after 42 d on regeneration medium with 5 μ M BA (bar = 1.2 cm). D, Callus produced on 'Marion' leaf after 63 d on regeneration medium with 10 μ M BA and 0.5 μ M IBA (bar = 0.8 cm).

TABLE 2

CAULOGENESIS OF BLACKBERRY WHOLE-LEAF EXPLANTS IN RESPONSE TO SEQUESTRENE IRON IN THE FIRST OR SECOND TRANSFER ON REGENERATION MEDIUM (RM)

IBA conc. (μ M)	RM ^a /T1	RM/T2	Caulogenesis ^b (%)		Callus index ^c
0.5			28.33 yz	3.36 yz	2.77 y
	+	-	25.00 y	1.96 y	2.70 y
	-	+	41.67 z	3.21 yz	2.73 y
	-	-	26.67 y	3.95 z	2.83 y
	+	+	15.00 xy	1.70 y	3.98 z
	+	-	21.67 xy	1.72 y	4.75 z
	-	+	16.67 xy	1.50 y	4.72 z
	-	-	10.00 x	1.47 y	4.73 z

^a Regeneration medium (RM) is NCCR-RUB medium with 2.9 μ M GA₃, 10 μ M BA, and solidified by 0.19% Gelrite. RM/T1 (first transfer to RM), RM/T2 (second transfer to RM). + or - = with or without sequestrene iron (200 mg l⁻¹).

^b Means with different letters in a column are significantly different at $P \leq 0.05$.

^c Callus index ranged from 0 (no callus) to 5 (81–100% callus on the leaf surface).

Effect of silver nitrate on regeneration. Silver nitrate did not improve the frequency of caulogenesis for 'Chester Thornless', 'Kotata', 'Marion', and 'Navaho' blackberries (data not shown). Significantly less callus ($P \leq 0.05$) was produced in explants grown on RM/T1 with silver nitrate compared to other treatments with or

without IBA, but there was no influence on caulogenesis. Cultivar differences were significant for the frequency of caulogenesis, shoots per explant, and callus index ($P \leq 0.001$). Regardless of the IBA content, 'Kotata' produced significantly more shoots than the other three cultivars, and more callus than 'Chester Thornless' and

'Navaho' on the 0.5 μ M IBA treatments. IBA did not influence the frequency of caulogenesis or the number of shoots per explant in any of the cultivars, but did induce more callus in all of them ($P \leq 0.05$). Callus formation was significantly affected by cultivar \times IBA concentration \times silver nitrate interaction ($P \leq 0.001$). Hyde and Phillips (1996) found that for chile pepper (*Capsicum annuum* L.), silver nitrate promoted caulogenesis in the shoot elongation stage; however, this was not observed for blackberry caulogenesis in this study. A significant reduction ($P \leq 0.001$) in callus formation was observed on explants subjected to silver nitrate in RM/T1, but no improvement in caulogenesis was observed with any treatment.

Rooting of adventitious shoots was spontaneous when shoots were transferred to medium without growth regulators or with only IBA (100%). Adventitious shoots appeared to arise directly from the leaf explants, but callus was sometimes present (Fig. 1). We did not study the histology of the shoot origin; however, the growth regulator combinations we applied did not appear to induce adventitious shoots via callus.

CONCLUSIONS

The three gelling agent treatments tested did not produce significant differences in the frequency of caulogenesis, shoots per explant, or callus formation for the blackberry genotypes tested, but the greatest frequency of caulogenesis and the most shoots per explant occurred on Gelrite RM with 10 μ M BA. Shoot production from explants of 'Kotata' and 'Marion' blackberries was significantly improved by adding sequestrene iron in the second transfer to RM. Silver nitrate significantly reduced callus formation in four cultivars and may be useful in promoting direct organogenesis in other *Rubus* cultivars or reducing callus production during micropropagation. IBA stimulated callus but did not increase shoot production per explant or the frequency of caulogenesis.

ACKNOWLEDGMENTS

We would like to thank Dr. Daryl G. Richardson, Department of Horticulture, Oregon State University (OSU) for loaning and instructing in the use of the fruit firmness tester and Dr. David Thomas of Department of Statistics, OSU, for his advice on data analysis.

REFERENCES

- Anderson, W. C. Tissue culture propagation of red and black raspberry, *R. idaeus* and *R. occidentalis*. Acta Hort. 112:13–20; 1980.
- Chevreau, E.; Mourgues, F.; Neveu, M.; Chevalier, M. Effect of gelling agents and antibiotics on adventitious bud regeneration from *in vitro* leaves of pear. In Vitro Cell. Dev. Biol. Plant 33:173–179; 1997.
- Cousineau, J. C.; Donnelly, D. J. Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss. Organ Cult. 27:249–255; 1991.
- Hyde, C. L.; Phillips, G. C. Silver nitrate promotes shoot development and plant regeneration of chile pepper (*Capsicum annuum* L.) via organogenesis. In Vitro Cell. Dev. Biol. Plant 32:72–80; 1996.
- Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.
- Reed, B. M. Multiplication of *Rubus* germplasm *in vitro*: a screen of 256 accessions. Fruit Varieties J. 44:141–148; 1990.
- SAS Institute, Inc. SAS user's guide, version 6. 2nd edn. Cary, NC: SAS Institute, Inc.; 1993.
- Swartz, H. J.; Bors, R.; Mohamed, F.; Naess, S. K. The effect of pretreatment on subsequent shoot organogenesis from *Rubus* and *Malus* leaves. Plant Cell Tiss. Organ Cult. 21:179–184; 1990.
- Taylor, P. W. J.; Ko, H. L.; Fraser, T. A.; Masel, N.; Adkins, W. Effect of silver nitrate on sugarcane cell suspension growth, protoplast isolation, ethylene production and shoot regeneration from cell suspension cultures. J. Exp. Bot. 45:1163–1168; 1994.
- Zimmerman, R. H.; Bhardwaj, S. V.; Fordham, I. M. Use of starch-gelled medium for tissue culture of some fruit crops. Plant Cell Tiss. Organ Cult. 43:207–213; 1995.