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Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry

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Abstract

Adventitious shoot regeneration was observed using leaf-petiole explants from shoot-proliferating cultures of 'Comet' red raspberry (*Rubus idaeus* L.). A maximum regeneration rate of 70% (3.7 shoots/explant) was obtained using 4.5–9.1 μM (1–2 mg l^{-1}) N-phenyl-N′-1,2,3-thiadiazol-5-ylurea (thidiazuron or TDZ) with 2.5–4.9 μM (0.5–1 mg l^{-1}) 1*H*-indole-3-butanoic acid (IBA) or 2.3 μM (0.5 mg l^{-1}) TDZ with 4.9 μM (1 mg l^{-1}) IBA in modified Murashige-Skoog medium. TDZ was more effective than *N*-(phenylmethyl)-1*H*-purin-6-amine (BA) at promoting regeneration in combinations tested with IBA (maximum 50% regeneration rate; 1.8 shoots/explant). Variation in the agar concentration or incubation temperature, orientation or scoring of the leaf-petiole explants and use of separate leaf or petiole explants had no effect on shoot regeneration. Incubation in the dark for 1, 2 or 3 weeks prior to growth in the light did not influence the percent regeneration rate but depressed the number of adventitious shoots. Explant source, from micropropagated shoots or greenhouse-grown plants, had an effect on shoot regeneration that was genotype dependent. Only 8 of 22 (36%) raspberry cultivars were capable of regeneration from leaf explants derived from greenhouse-grown plants.

Introduction

Genetic transformation of plants with *Agrobacterium* vectors requires efficient adventitious shoot regeneration systems. Adventitious shoot regeneration was reported from blackberry cotyledons and leaves (Fiola & Swartz 1986; Fiola et al. 1990), from blackberry × raspberry hybrids (Swartz et al. 1990) and also, occasionally, from raspberry leaf petioles or lamina in contact with the medium in axillary shoot cultures (Feucht et al. 1985). Adventitious shoot regeneration also occurred on leaf discs and internodal stem segments from micropropagated cultures of two red raspberry genotypes and blackberry (McNicol & Graham 1990). The regeneration frequency of the raspberry genotypes was low (from 7.5 to 38% depending on genotype and explant type used) but higher (64%) for one genotype if internodal stem segments were peeled. Although Graham et al. (1990) were able to achieve *Agrobacterium*-mediated transformation of raspberry using peeled internodal stem segments, this type of explant is not ideal for genetic transformation studies.

Our objective was to develop an efficient system for adventitious shoot regeneration from raspberry leaf tissues by manipulating media components, explant tissue types and incubation conditions. In addition, explants obtained from micropropagated shoots were compared to those

obtained from greenhouse-grown plants and the effect of raspberry genotype on adventitious shoot regeneration was determined.

Materials and methods

Red and purple raspberry (*Rubus idaeus* L. and *R.* × *neglectus* Peck) cultivars were maintained in the greenhouse at ambient temperatures under natural daylight. Tissue cultured shoots were derived from shoot tips of greenhouse-grown plants. They were aseptically cultured on a Murashige-Skoog (MS) medium (1962) modified for *Rubus* by Donnelly et al. (1986) with 4.4 μM (1.0 mg l^{-1}) *N*-(phenylmethyl)-1*H*-purin-6-amine (BA), 0.5 μM (0.1 mg l^{-1}) 1*H*-indole-3-butanoic acid (IBA), and elevated levels of thiamine. HCl (2 mg l^{-1}). The medium was solidified with 6 g l^{-1} Anachemia agar and the cultures were maintained at 25°C under a 16-h photoperiod of cool-white fluorescent light at 50.4 μmol m^{-2} s^{-1}.The shoots were subcultured onto fresh medium every 3–4 weeks.

Growth regulator factorials

Leaf-petiole explants (0.5 to 1.5 cm^2 leaves with half the petiole attached) were obtained from micropropagated axillary shoot cultures of 'Comet' red raspberry and placed in 100 × 15 mm petri dishes containing 25 ml *Rubus* medium described above. Two growth regulator factorial combinations were evaluated for promotion of adventitious shoot regeneration: BA at 0, 2.2, 4.4, 8.9 and 17.8 μM (0, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}) and N-phenyl-N′-1,2,3-thiadiazol-5-ylurea (thidiazuron or TDZ) at 0, 2.3, 4.5, 9.1 and 18.2 μM (0, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}) each with IBA at 0, 0.5, 2.5 and 4.9 μM (0, 0.1, 0.5 and 1.0 mg l^{-1}). The leaf-petiole explants were placed with the adaxial surface toward the medium. Each growth regulator factorial combination was evaluated twice using five explants/plate and five plates/treatment. The cultures were incubated in a growth chamber at 21°C under a 16-h photoperiod of cool-white fluorescent light at 50.4 μmol m^{-2} s^{-1}. After 6 weeks incubation, the percent explant regeneration and the average number of shoots ≥1 mm long per regenerating explant were determined. Data from each set of two experiments were pooled when homogeneity of the experimental error was confirmed using the F test. The orthogonal polynomial method was used to determine the functional relationship between response and treatment. Percent explant regeneration data were transformed to arcsin of square-root and shoot number data were transformed to square-root ($X + 0.5$) prior to statistical analysis.

Conditions affecting regeneration from tissue culture-derived explants

Leaf-petiole explants of 'Comet' were used to investigate the effect of agar concentrations (2, 4, 8, 10 g l^{-1}), higher incubation temperature (25°C), and dark incubation (initial 1, 2 or 3 weeks of the 6-week total) on adventitious shoot production in *Rubus* regeneration medium containing 4.5 μM (1.0 mg l^{-1}) TDZ and 2.5 μM (0.5 mg l^{-1}) IBA. The following explant modifications were also evaluated for their effect on adventitious shoot regeneration in the same medium: placing the leaf-petiole explant abaxial side down, scoring (wounding) the explant once through the mid-vein of the lamina, using a leaf without a petiole portion, and using isolated petioles (0.5–1.0 cm long). In control cultures leaf-petiole explants were placed adaxial side down on *Rubus* regeneration medium solidified with 6 g l^{-1} agar and incubated at 21°C for 6 weeks under a 16-h photoperiod. All experiments were repeated twice using five explants/plate and five plates/treatment. Duncan's new multiple range test was used on transformed data to separate the treatments for percent explant regeneration and mean number of shoots ≥1 mm long per regenerating explant.

In a separate experiment, leaf squares (0.16 to 0.36 cm^2) cut from micropropagated shoots of 'Comet' were compared to leaf-petiole explants obtained from similar material and treated as the control plates above.

Explants from tissue cultured vs greenhouse-grown plants

Leaf-petiole explants from micropropagated

shoots and 0.25 cm^2 squares cut from greenhouse-grown leaves of the cultivars Algonquin, Comet, Festival, Heritage, Killarney and Titan were plated as described in the growth regulator factorials on *Rubus* regeneration medium containing 4.5 μM (1.0 mg l^{-1}) TDZ, 2.5 μM (0.5 mg l^{-1}) IBA and 7 g l^{-1} agar. Prior to plating, the greenhouse-grown leaves were disinfested by rinsing for 30 min under running water followed by a 15 min incubation with frequent agitation in 10% bleach (commercial preparation containing 5.25% sodium hypochlorite) and two rinses of 5 min each in sterile distilled water. The experiment was repeated twice using five explants/plate and five plates/treatment and Duncan's new multiple range test was used on transformed data to separate the treatments.

Effect of genotype on the regeneration of greenhouse-derived explants

The adventitious shoot regeneration response was tested twice using five explants/plate and five plates/treatment for the following 14 red and 2 purple raspberry cultivars: Boyne, Brandywine (purple), Carnival, Chilcotin, Citadel, Comox, Glen Prosen, Latham, Madawaska, Meeker, Newburgh, Royalty (purple), Scepter, Skeena, Southland and spine-free Willamette. Leaf explants 0.25 cm^2 in size from greenhouse-grown plants were incubated as described in the growth regulator factorial experiments on *Rubus* regeneration medium containing 4.5 μM (1.0 mg l^{-1}) TDZ, 2.5 μM (0.5 mg l^{-1}) IBA and 7 g l^{-1} agar.

Results and discussion

Growth regulator factorials

Explants on media lacking the cytokinins TDZ (Fig. 1) or BA (data not shown) did not regenerate adventitious shoots. TDZ promoted a greater percentage shoot regeneration and more shoots/explant than BA. A maximum of 70% regeneration (3.7 shoots/explant) was obtained with 1 mg l^{-1} TDZ and 0.5 mg l^{-1} IBA compared to a

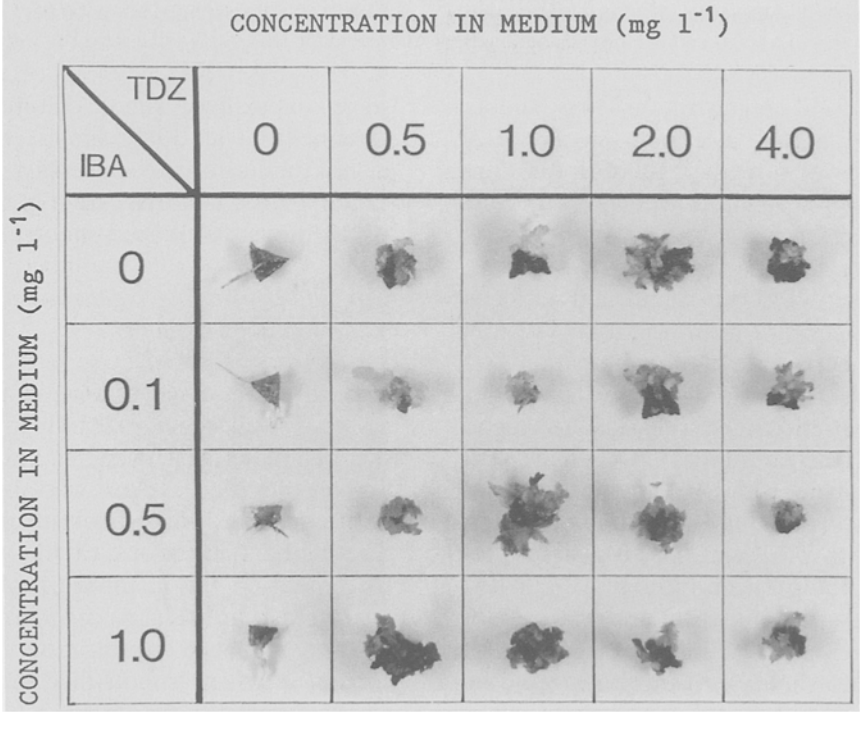

Fig. 1. The effect of TDZ (0, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}) and IBA (0, 0.1, 0.5 and 1.0 mg l^{-1}) growth regulators on adventitious shoot formation using 'Comet' leaf-petiole explants obtained from axillary shoot cultures.

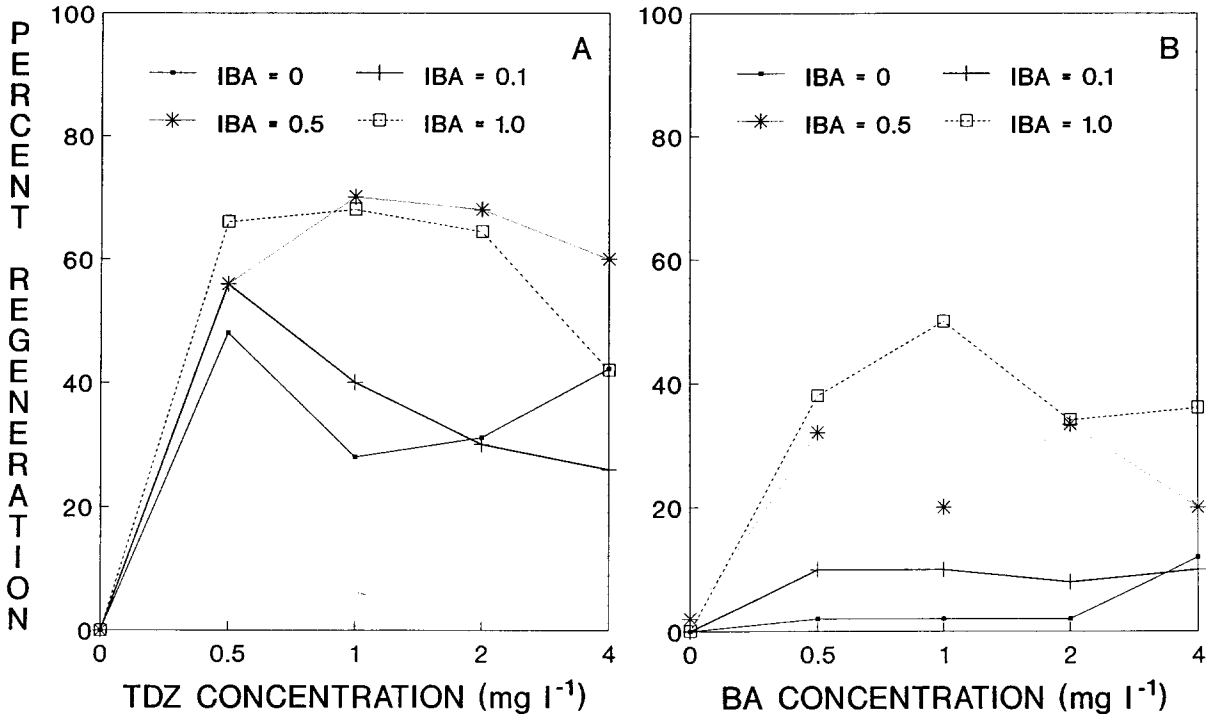

Fig. 2. Percent regeneration of 'Comet' leaf-petiole explants obtained from axillary shoot cultures. (*A*) TDZ and IBA growth regulator factorial. TDZ and IBA main effects were significant at $p < 0.01$ and there was a significant TDZ × IBA interaction ($p < 0.05$). The response of TDZ was cubic at all levels of IBA ($p < 0.01$). (*B*) BA and IBA growth regulator factorial. BA and IBA main effects were significant at $p < 0.01$ and there was a significant BA × IBA interaction ($p < 0.01$). The response of BA was not significant for IBA = 0.0 and 0.1 mg l^{-1}, and quadratic and cubic for IBA = 0.5 and 1.0 mg l^{-1}, respectively ($p < 0.01$).

maximum of 50% regeneration (1.8 shoots/explant) with 1 mg l^{-1} BA and 1 mg l^{-1} IBA. The best ranges of growth regulators for shoot regeneration were 1–2 mg l^{-1} TDZ with 0.5–1 mg l^{-1} IBA and 0.5 mg l^{-1} TDZ with 1 mg l^{-1} IBA. The average number of shoots per regenerating explant was not significantly affected by the various levels of growth regulators within each cytokinin/auxin factorial combination.

On media with cytokinins, explants regenerated shoots better in the presence of higher levels of IBA (0.5 and 1 mg l^{-1}) (Fig. 2). This was especially true with BA and less so with TDZ although explant regeneration was significantly lower with TDZ and 0 or 0.1 mg l^{-1} IBA than at higher IBA concentrations.

Although BA was not as effective at promoting regeneration as TDZ, an average of 10% regeneration (1.6 shoots/explant) occurred using medium containing 1 mg l^{-1} BA and 0.1 mg l^{-1} IBA. These same growth regulator concentrations were recommended for the multiplication stage of axillary shoot cultures of raspberry (Donnelly et al. 1980; James et al. 1980). Our results indicate the necessity for monitoring axillary shoot cultures carefully to rogue out any adventitiously produced shoots.

Conditions affecting regeneration from tissue culture-derived explants

The adventitious shoot regeneration rate (average 65%) was not significantly affected by any of the treatments tested and the number of shoots per regenerating explant was also unaffected by agar concentration, higher incubation temperature and modifications to the explant type or placement on the medium (Table 1). However, an increase in the agar concentration from 6 to 8 mg l^{-1} reduced vitrification of regenerated shoots. Vitrified shoots had translucent stems and leaves that were thickened, turgid and brittle. Higher agar concentrations also reduced vitrification in other tissue culture systems (Ziv et

Table 1. Conditions affecting regeneration from tissue culture derived explants of 'Comet'.

Explant	Agar ($g\,l^{-1}$)	Temperature (°C)	Incubation[1]		Side down		Mean number shoots/ regenerating explant[2]
			Dark	Light	Adaxial	Abaxial	
Leaf-petiole	2	21	0	6	+		5.2 abc
Leaf-petiole	4	21	0	6	+		4.9 abcd
Leaf-petiole	8	21	0	6	+		4.5 abcd
Leaf-petiole	10	21	0	6	+		3.9 bcd
Leaf-petiole	6	25	0	6	+		4.6 abcd
Leaf-petiole	6	21	1	5	+		3.4 cd
Leaf-petiole	6	21	2	4	+		3.3 cd
Leaf-petiole	6	21	3	3	+		2.8 d
Leaf-petiole	6	21	0	6		+	4.8 abcd
Scored leaf-petiole	6	21	0	6	+		6.2 ab
Leaf	6	21	0	6	+		6.3 a
Petiole	6	21	0	6	+		5.2 abc
Leaf-petiole (control)	6	21	0	6	+		6.0 ab

[1] Number of weeks of incubation in the dark followed by incubation in the light.
[2] Mean separation in a column by Duncan's new multiple range test at 5% level on square-root ($X + 0.5$) transformed data. Figures represent the untransformed means from two pooled repetitions.

al. 1983). An agar concentration of $7\,mg\,l^{-1}$ was used in all subsequent experiments. The average number of shoots per regenerating explant was reduced in plates incubated for 1, 2, or 3 weeks in the dark prior to growth in the light. This may have occurred because preliminary incubation in the dark delayed shoot initiation.

No differences were seen in the adventitious shoot regeneration rate and the number of shoots per regenerating explant for leaf squares cut from tissue cultured shoots of 'Comet' compared with leaf-petiole explants.

Explants from tissue cultured vs greenhouse-grown plants

The organogenic potential of explants from tissue cultured shoots compared with those taken from greenhouse-grown plants was genotype dependent (Table 2). 'Algonquin' and 'Comet' had better regeneration rates using explants from tissue culture (46.7 and 76.0% respectively) compared with greenhouse-derived explants (2.0 and 50.0%). The reverse was observed for 'Heritage', which regenerated from greenhouse material (37.8%) but not at all from tissue culture-derived explants. 'Festival', 'Killarney', and 'Titan' explants obtained from both sources did not regenerate. Such differences in regeneration between tissue cultured and greenhouse-grown material were also reported by Liu & Sanford (1988) for strawberry. They found that different concentrations of growth regulators were necessary to optimize regeneration from both types of explants.

Table 2. Shoot regeneration comparison between leaf-petiole explants derived from tissue cultured shoots and 0.25 cm^2 leaf explants from greenhouse-grown plants of six cultivars.

Explant source	Percent explant regeneration[1]	Shoots ≥ 1 mm/regenerating explant[1]
	Tissue culture	
Algonquin	46.7 b	3.1 bc
Comet	76.0 a	4.8 abc
Festival	0.0 c	–
Heritage	0.0 c	–
Killarney	0.0 c	–
Titan	0.0 c	–
	Greenhouse	
Algonquin	2.0 c	1.0 d
Comet	50.0 b	9.9 a
Festival	0.0 c	–
Heritage	37.8 b	1.6 bc
Killarney	0.0 c	–
Titan	0.0 c	–

[1] Data analysed by Duncan's new multiple range test on arcsin (square-root X) (percent regeneration) or square root ($X + 0.5$) (shoots/regenerating explant) transformed data. Untransformed means shown. Responses within columns are not significantly different ($p = 0.05$) if the same letter appears.

Effect of genotype on the regeneration of greenhouse-derived explants

'Citadel' and 'Southland' had the highest adventitious shoot regeneration rate (both with 44.4%; 2.0 and 1.7 shoots/explant respectively) followed by 'Madawaska' (12.0%; 1.0 shoot/explant), 'Carnival' (8.0%; 1.5 shoots/explant), and 'Brandywine' (6.7%; 1.0 shoot/explant). The following cultivars did not regenerate: Boyne, Chilcotin, Comox, Glen Prosen, Latham, Meeker, Newburgh, Royalty, Scepter, Skeena, and spine-free Willamette. By including the results obtained for the cultivars tested in the previous experiment a total of 8 of 22 (36%) cultivars were capable of shoot regeneration. Similar low adventitious shoot regeneration was noted in other aspects. Mikami et al. (1989) reported that direct shoot formation from beet (*Beta vulgaris* L. and *B. maritima* L.) leaf discs was limited to 2 of 9 (22%) genotypes tested. Only 28 of 85 (33%) lines and cultivars of cucumber (*Cucumis sativus* L.) formed shoots from cotyledon explants (Wehner & Locy 1981). Since explants from tissue-cultured shoots responded differently to those taken from greenhouse-grown plants, it is hoped that some of the cultivars with low or no adventitious shoot regeneration using greenhouse-derived explants may regenerate better by using explants obtained from micropropagated shoot cultures. The use of pretreatments of source plants using 150 μM colchicine for three days followed by treatment with 0.5 to 1.0 μM TDZ for three weeks prior to leaf excision has been shown to enhance regeneration in two blackberry-raspberry hybrids (Swartz et al. 1990). This type of pretreatment may also be beneficial in promoting regeneration in raspberry cultivars that presently show no regeneration.

An efficient adventitious shoot regeneration system was developed from 'Comet' red raspberry leaf-petiole explants using 1–2 mg l^{-1} TDZ with 0.5–1 mg l^{-1} IBA or 0.5 mg l^{-1} TDZ with 1 mg l^{-1} IBA in MS medium. This system resulted in a regeneration rate that was sufficiently high (70%) and with sufficient shoots/explant (3.7) to be useful for future *Agrobacterium*-mediated transformation work. There was a marked effect of genotype on the ability to regenerate shoots adventitiously and this effect varied depending on whether explants originated from micropropagated shoots or greenhouse-grown plants.

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