식물생명공학회지 제35권 제2호
J Plant Biotechnol
Vol. 35, No. 2, 163-167 (2008)

The identification of optimum condition for direct regeneration in black raspberry

Choi Heh Ran[1], Pill Jae Park[1], Hee Kwon Lee[1], Yun Song Joong[2], and In Sok Lee[1*]

[1]Black Raspberry R&D Team, Gochang Agricultural Extension Center, Gochang, Korea

[2]College of Agricultural and Life Science, Chonbuk National University, Jeonju, Korea

ABSTRACT Adventitious buds appeared within 2 weeks on the base of the petiole explants and increased for two months. A maximum of regeneration (15.6%) was obtained on the medium containing 1.5 μM TDZ in combination with 1 μM IBA. To know which explants are the best for the induction of regeneration, three explants such as leaf, petiole and leaf-petiole were used. Among the explant types, the leaf-petiole explant was significantly more effective than leaf and petiole for promoting adventitious shoots, with leaf-petiole inducing at the highest regeneration frequency (33.7%). The regeneration frequency of adventitious shoots in the leaf-petiole explants was significantly affected by leaf size and the position of explants. The leaf-petiole smaller than 5 mm leaf in width was induced at the highest regeneration frequency (68.9%). The smaller leaf sizes, the greater regeneration frequency. Also when the leaves are nearer to the shoot tip, the regeneration frequency is higher. When the rooted micro-shoots were transferred to the soil after growing for 6 weeks in the media, the survival rate was 90%.

Introduction

The Korean black raspberry (*Rubus coreanus* Miq.) is a perennial shrub which is distributed in southern part of Korea, it has been used traditionally for medicined purposes and primarily consumed as fresh fruit and processed food at present, also is a crop of great importance to make a large sum of money for farmers in some regions.

Healthy, vigorous and true-to-mane planting stock of black raspberry is essential for successful propagation. It is propagated by vegetative methods including root buds, tip layers, root cuttings and tissue culture can obtain many plantlets such as virus-free and fungus-free stocks at the same time. The stem diameter of healthy vegetative clone is ranging from 6 to 9 mm. Larger roots and clones are not suitable because of spacing the root buds farther apart and not having any viable buds present. However, the tissue culture can be used in obtaining reliable and uniform nursery stocks all year round and fast. So, the tissue culture can be remarkably useful to make use of the technique combined with radiation for the breeding of black raspberry. Also, *In vitro* plant regeneration is the most important resources for biotechnological application except for plant improvement programs.

In Rubus, there were many reports on rapid regeneration and multiplication by organogenesis via callus formation. The rapid regeneration and multiplication was reported from cotyledons and leaves (Fiola and Swartz 1986, Fiola et al. 1990, Swartz et al. 1990, Owensy de Novoa and Conner 1992, Gingas and Stokes 1993), from blackberry X 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, Cousineau and Donnelly 1991). Also rapid adventitious shoot regeneration occurred on leaf discs and internodal stem segments from micropropagated cultures of two red raspberry genotypes and blackberry

*Corresponding author Tel 063-564-0111 Fax 063-560-2537
E-mail: insoklee@hanmail.net

(McNicol and Graham 1990). However, few dealt with direct regeneration from different explants.

The present study is the first report on direct shoot regeneration of Korean black raspberry. The purpose of this study is to investigate the optimum condition for *in vitro* propagation using direct induction of shoot buds from leaf explants of *in vitro*-raised shoots in Korean black raspberry .

Materials and methods

Plant materials

The Korean black raspberry (*Rubus coreanus* Miq.) cultivated in Gochanggun was maintained in the greenhouse under natural daylight. Shoots for *in vitro* culture were derived from shoot tips of greenhouse-grown plants. They were aseptically cultured on the MS media supplemented with 4.4 μM benzyladenine (BA) and 1.0 μM indolebutyric acid (IBA) to obtain many leaf-petiole tissues. The pH was adjusted to 5.8 before autoclaving at 1.1 $kg.cm^{-2}$ and autoclaved at 121℃ for 20 min. The cultures were incubated at a photosynthetic photo flux density (PPFD) of 20 ± 5 $\mu mol^{-2}s^{-1}$ from cool white fluorescent lamps at 25 ± 1℃ with a 16h light/8h dark cycle. The shoots were subcultured onto fresh media every 4 weeks.

Effect of growth regulators for regeneration

One-month-old shoots of black raspberry were excised aseptically from *in vitro*-grown cultures. One shoot was used to provide leaves for each Petri dish. The expanding leaves were excised from the shoot apex, removing the petioles. The two pieces of 1 cm in width and 1 cm in length were made through the midvein on the abaxial side. The leaf explants were placed on media containing 0.5, 1.5, 2.5, 5.0 and 10 μM TDZ in combination with 0, 0.5, 1.0 and 2.5 μM IBA with three replicates on the base of 10 Petri dishes per replicate, containing 5 explants per Petri dish. The Petri dishes were placed at 25 ± 1℃ in the dark for 3 days before transferring to the light as described previously. The number of leaves forming shoot were recorded after 4 and 8 weeks. Adventitious buds were counted as shoots. Data were analyzed using the General Linear Models Procedure of SAS (SAS Institute Inc. 1987).

Effect of explant type and leaf size on adventitious shoot regeneration

The various explants such as leaf, petiole and leaf-petiole were used to investigate the effect on adventitious shoot regeneration on medium containing 1.5 μM TDZ in combination with 1.0 μM IBA. Leaf-petiole explants were classified into three categories of LP-1 (5 mm below leaf in diameter), LP-2 (5~10 mm leaf in diameter) and LP-3 (10~15 mm leaf in diameter). The explants were inoculated on the media with three replicates on the base of 10 Petri dishes per replicate, containing 5 explants to each Petri dish. The Petri dishes were placed at 25 ± 1℃ in the dark for 3 days before transferring to the light as described previously.

The number of leaves formed from shoots and the number of shoots formed from a leaf were recorded after 8 weeks *in vitro* condition. Adventitious buds were counted as shoots. Data were analyzed using the General Linear Models Procedure of SAS (SAS Institute Inc. 1987).

Rooting and acclimatization

Rooting was induced on the MS medium supplemented with 1.0 μM IBA in the dark for 1 week. The micro-shoots were later transferred to hormone-free MS medium. The rooted micro-shoots were transferred to Hikko tray/pots containing vermiculite:perlite (7:3) in the specially-designed hardening chamber for 4 weeks and later transferred to larger pots (20 cm diameter) in a greenhouse with sand:garden soil:vermiculite (1:1:1) for further growth.

Results and discussion

Effect of growth regulators on regeneration

Before the induction of adventitious buds, some shoot tips derived from plants grown outside were cultured *in vitro* condition to propagate leaf for one month. Adventitious buds were observed directly on the base of the petiole explants

within 2 weeks and their number were continuously increased for two months. In combination of TDZ and IBA, regeneration was not shown in the treatment of TDZ alone without IBA (Table 1). It is apparent that the interaction of TDZ and IBA was considerably important for the regeneration, showing a different regeneration pattern in each combination of TDZ and IBA. A maximum of regeneration (15.6%) was obtained on the medium containing 1.5 μM TDZ in combination with 1 μM IBA after 8 weeks (Table 1). Regeneration frequency decreased with increasing TDZ concentration. In each combination of 10 μM TDZ and all ranges of IBA, there are no plantlets (Table 1). It is thought that a high TDZ is not suitable for regeneration. The marked effect of the combination of growth regulators on adventitious shoot regeneration was reported by Cousineau and Donnelly (1991) and Pratap et al. (2004).

Table 1 Effect of TDZ in combination with IBA concentrations for regeneration frequency using black raspberry leaves

TDZ (μM)	IBA (μM)	Regeneration (%) 4 weeks	Regeneration (%) 8 weeks
0.5	0	0.0 d[y]	0.0 f
	0.5	3.3 bcd	4.4 bcde
	1	3.3 bcd	5.6 bcde
	2.5	3.3 bcd	5.6 bcde
1.5	0	0.0 d	0.0 f
	0.5	4.4 abc	6.7 bcde
	1	7.8 abc	15.6 a
	2.5	5.6 abc	7.8 bcde
2.5	0	0.0 d	0.0 f
	0.5	2.2 bcd	4.4 bcde
	1	2.2 bcd	5.6 bcde
	2.5	2.2 bcd	3.3 cdef
5	0	0.0 d	0.0 f
	0.5	0.0 d	1.1 ef
	1	1.1 cd	2.2 def
	2.5	0.0 d	0.0 f
10	0	0.0 d	0.0 f
	0.5	0.0 d	0.0 f
	1	0.0 d	0.0 f
	2.5	0.0 d	0.0 f

[y] Values for TDZ and IBA concentrations within each column followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Rang test.

Effect of explant type and leaf size

To identify which explants is good for the induction of plant-lets, a variety of explants (leaf, petiole and leaf-petiole) were inoculated and adventitious shoots were emerged directly from the cut surfaces of explants after 2 weeks. Among the explant types, leaf-petiole has induced the highest regeneration frequency (33.7%) after 8 weeks. The leaf-petiole explant was significantly more effective than other explants such as leaf and petiole for promoting adventitious shoots (Table 2). As found by Fasolo *et al.* (1989) and Welander & Maheswaran (1992) for apples, tissue selection was an important factor in obtaining high frequency regeneration.

The adventitious shoot regeneration rate was significantly affected by leaf size of leaf-petiole tissue and regeneration capacity was decreased with a leaf being bigger in diameter (Figure 1, Left). The regeneration frequency (68.9%) of leaf-petiole of 5 mm below leaf in diameter was about three times higher than that (25.6%) of 5~10 mm leaf and about 10 times greater than that (6.7%) of 10~15 mm leaf (Figure. 1, Left). No significant difference was observed in number of shoots formed from each leaf-petiole tissue (Figure. 1, Right). This technology can be useful for maintaining clonal fidelity of elites. A similar results on the importance of tissue selection for regeneration was also reported by Dubois and de Vries (1995), Antonelli and Druart (1990), Cousinneau and Donnelly (1991), Escalettes and Dosba (1993). The regenerative capacity at the base of petiole, however, could be ascribed to (1) basipetal transport of endogenous auxins and/or carbohydrates

Table 2 Effect of explants for regeneration frequency and number of shoots per regenerated explant on the medium containing 1.5 μM TDZ in combination with 1.0 μM IBA in black raspberry

Kind of sources	A	B	C
Leaf-petiole	23.7 a	33.7 a	7.3 a
Petiole	17.0 b	23.0 b	7.1 a
Leaf	4.4 c	7.4 c	6.9 a

[y] Values for TDZ and IBA concentrations within each column followed by the same letter are not significantly different at the 5% level by Duncan's Multiple Rang test.
A: Regeneration frequency (%) after 4 weeks, B : Regeneration frequency (%) after 8 weeks, C : No. of shoots per regenerated explants after 8 weeks.

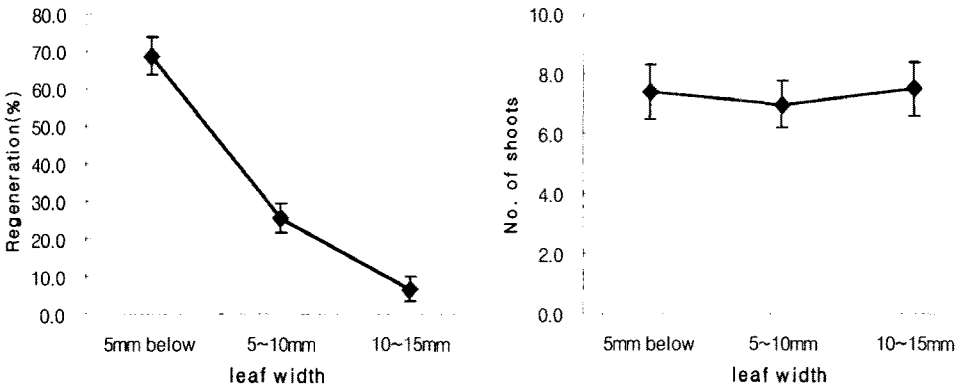

Figure 1. Comparison of regeneration frequency and shoot number depending on leaf size of leaf-petiole tissue after 8 weeks in black raspberry. Left : Regeneration frequency (%), Right : No. of shoots per regenerated explants, leaf-petiole. Vertical bars represent ±SE.

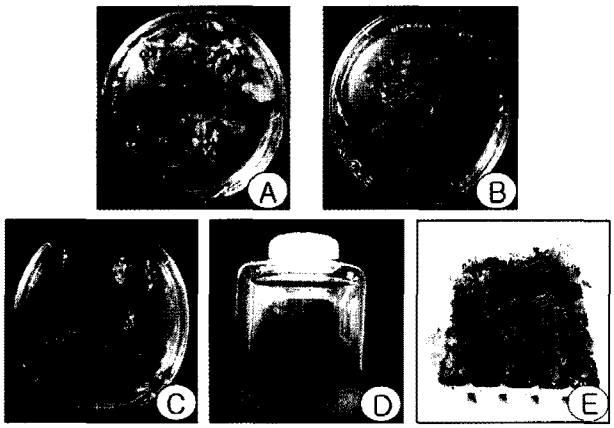

Figure 2. Adventitious shoot formation and plant regeneration in tissue cultures in black raspberry. (A) Explant obtained after 4 weeks from axillary shoot culture; (B) Leaf-petiole tissue obtained from A to regenerate plantlet; (C) Adventitious shoots formed on petiole explant; (D) Complete regenerated plantlet on rooting media; (E) Regenerated plant after four weeks of transplantation.

(Dubois and de Vries, 1995) or (2) position of the regenerative target cells (Margara, 1982).

Rooting and acclimatization

The proliferated micro-shoots, when transferred to rooting medium, produced roots within 2 weeks (Figure 2, D). The rate of root induction was found to be 80% after 3 weeks of culture (Figure 2, D). When the rooted micro-shoots were transferred to the soil after 6 weeks induction in culture, showed 90% survival rate (Figure 2, E).

The present study reports the development of a protocol causeing direct regeneration from the youngest expanding leaves near the shoot apex *in vitro*.

Acknowledgement

This work was supported by a grant from On-Site Cooperative Agriculture Research Project, Rural Development Administration, South Korea.

References

Antonelli M, Druart P (1990) The use of a brief 2,4-D treatment to induce leaf regeneration on *Prunus canescens* Bois. Acta Hort 280: 45-50

Cousineau JC, Donnelly DJ (1991) Adventitious shoot regeneration from leaf explants of tissue cultured and greenhouse-grown raspberry. Plant Cell Tiss Org Cult 27: 249-255

Dubois LAM, de Vries DP (1995) Preliminary report on direct regeneration of adventitious buds on leaf explants of *in vivo* grown glass house rose cultivars. Gartenbauwissenschaft 60: 249-253

Escalettes V, Dosba F (1993) *In vitro* adventitious shoot regeneration from leaves of *Prunus spp.* Plant Sci Limerick 90: 201-209

Fasolo F, Zimmerman RH, Fordham I (1989) Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars. Plant Cell Tiss Org Cult 16: 75-87

Feucht W, Schmid PPS, Schimmelpgeng H, Theiler-Hedtrich C, Schmitt A (1985) In vitro vermehrte himbeeren im groBversuch: Abweicherquote vom sortentypus von 0.68 prozent und enzyme-muster der atypischen fruchte. Erwerbsobstbau 27: 167-169

Fiola JA, Swartz HJ (1986) Somatic embryogenesis, organogenesis and proliferation in vitro from *Rubus* embryos. Acta Hort

183: 91-98

Fiola JA, Hassan MA, Swartz HJ, Bors RH, McNicols R (1990) Effect of thidiazuron, light fluence rates and kanamycin on in vitro shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss Org Cult 20: 223-228

Gingas VM, Stokes BD (1993) Rubus plat regeneration via asexual embryogenesis. HortScience 28: 58

Margara J (1982) Bases de la multiplication vegetative. Les meristemes et l'organogenese, INRA 149, Rue de grenelle, 75341 Paris Codex 07, France

McNicol RJ, Graham J (1990) In vitro regeneration of *Rubus* from leaf and stem segments. Plant Cell Tiss Org Cult 21: 45-50

Owensy de Novoa C, Conner AJ (1992) Comparison of in vitro shoot regeneration protocols from *Rubus* leaf explants. N. Z. J. Crop Hort Sci 20: 471-476

Pratap KP, Madhu S, Anil S, Ahuja PS (2004) Direct shoot regeneration from leaf explants of *Rosa Damascena* Mill. In Vitro Cell Dev Biol-Plant 40: 192-195

SAS Institute Inc (1987) SAS/STAT™ Guide for Personal Computers, Version 6 Edition. Cary NC SAS Institute Inc: 1028

Swatz HJ, Borsn R, Mohamed F, Naess SK (1990) The effect of in vitro pretreatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. Plant cell Tiss Org Cult 21: 179-184

Welander M, Maheswaran G (1992) Shoot regeneration from leaf explants of dwarfing apple rootstocks. J. Plant Physiol 140: 223-228

(Received March 4, 2008; Accepted April 21, 2008)