**EFFECTIVE MICROPROPAGATION OF RABBITEYE BLUEBERRIES FOR LEAF TEA PRODUCTION**

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**Abstract**

Micropropagation of two clones of rabbiteye blueberry (*Vaccinium virgatum* Aiton) developed for leaf tea production was investigated for supply in high quantities to plantations and plant factories. Shoots of a new cultivar, ‘Kunisato 35 Gou’, were multiplied efficiently on MW, a mixture of equal parts of MS and WPM, supplemented with 20 μM zeatin by using 2-node segments. For No. 19, another promising clone, 5-node segments were the most productive for shoot multiplication. Among basal media tested for micropropagation, MW medium was found to be the most efficient. Although shoots of ‘Kunisato 35 Gou’ grew better *in vitro* than those of No. 19, both clones had equivalent rooting abilities. More than 90% of the shoots rooted in vermiculite after culturing in Gellan gum-solidified rooting medium without plant growth regulators. The growth of shoots with roots in pots was significantly influenced by the pH of the potting soil. The plantlets in the mildly acidic soil grew vigorously, while those in the neutral soils grew moderately or poorly, depending on soil type.

**Key words:** MW medium, node number of segments, potting soil, *Vaccinium virgatum*

**INTRODUCTION**

Micropropagation is the most effective propagation system for new cultivars of blueberries because plenty of plants can be rapidly produced. Hence, micropropagation of blueberries has been intensively developed over the last 30 years (Debnath, 2009, Fukui et al., 1991, Gonzalez et al., 2000, Isutsa et al., 1994, Lyrene, 1980, Nickerson, 1978, Wolfe et al., 1986). Since Wolfe et al. (1983) showed that woody plant medium (WPM) (Lloyd and McCown, 1980) produced the best shoot growth among seven media tested for micropropagation of ‘Bluecrop’ highbush blueberries, most researchers have used WPM as a basal medium for blueberry micropropagation (Chandler and Draper, 1986, Fukui et al., 1991, Gonzalez et al., 2000, Isutsa et al., 1994, Reed and Abdelnour-Esquivel, 1991, Wolfe et al., 1986). However, we reported that WPM was not ideal for the micropropagation of four highbush blueberry cultivars, including ‘Bluecrop’, because the shoots grew better on MW, which is a mixture of equal parts of MS (Murashige and Skoog, 1962) and WPM, than on MS or on WPM (Tetsumura et al., 2008). The micropropagation of Japanese wild *Vaccinium* and some other blueberry cultivars has been successful when using MW medium (Sato-Yamauchi et al., 2012).

Blueberry fruit is well known for its potential health benefits, attributable mostly to its high content of antioxidant phenolic compounds: anthocyanins, flavonols, and phenolic acids. Recently, its leaves have also been found to contain antioxidant phenolic compounds, including proanthocyanidin, which may inhibit hepatitis C virus RNA expression (Takeshita et al., 2009). Among the *Vaccinium* cultivars from the section *cyanococcus* that were tested, rabbiteye blueberry (*V. virgatum* Aiton) leaves had the highest proanthocyanidin content (Yoshino et al., 2009). The application by the University of Miyazaki and Miyazaki Prefectural Industrial Support Foundation to the Plant Variety Protection Office at the Ministry of Agriculture, Forestry and Fisheries in Japan for registration of a new rabbiteye blueberry cultivar ‘Kunisato 35 Gou’ for leaf production was accepted in 2009. However, an effective method to propagate this new cultivar, which had been developed from a seedling, had not yet been investigated. If a micropropagation system for rapid multiplication of this cultivar is established, plenty of nursery stock will be available for plantations and plant factories, which require more plants per area than orchards. Moreover, improvement of the micropropagation system might create a new industry: *in vitro* blueberry leaf tea production.

In the present study, we developed a micropropagation system for the efficient multiplication, elongation, and rooting of shoots in ‘Kunisato 35 Gou’ and No. 19, another rabbiteye blueberry clone that has leaves with high antioxidant phenolic compounds. Efficient horticultural methods for raising plantlets into mature nursery plants for blueberry leaf tea plantations were also investigated.

**MATERIALS AND METHODS**

*In vitro* propagated shoots of ‘Kunisato 35 Gou’ and No. 19 were obtained from the original cultivar plants raised from seedlings according to Tetsumura et al. (2008). After several rounds of subculturing on MW medium supplemented with 20 μM 6-(4-hydroxy-3-methylbut-2-enylamino)purine (zeatin), the best multiplication medium with plant growth regulators for the four highbush blueberry cultivars (Tetsumura et al., 2008), the shoots were cut into five types of segments: 1-node with a leaf, 2-nodes with two leaves, 3-nodes with three leaves, 4-nodes with four leaves, and 5-nodes with five leaves. Four segments of the same type were placed in a 100-mL Erlenmeyer flask containing 20 mL medium. The medium was the same as that used for subculturing. The survival rate, the length of the longest shoot, and the number of useable shoots at least 10 mm long (Wolfe et al., 1983) were recorded 60 d after initiation of the culture. Each treatment consists of five flasks containing 20 segments, and these experiments were conducted four times.

The results of the experiment showed that the most efficient propagation was obtained using the 2-node segments of ‘Kunisato 35 Gou’ and the 5-node segments of No. 19. Therefore, these segments were used for the basal medium experiment. In preliminary trials, MS medium with 20 μM zeatin was used, but all of the shoots died. Subsequently, the shoots were planted on 8MW medium, a mixture of MS and WPM (8:2, v/v); half of the shoots died on this 8MW medium. Therefore, basal media with moderate ratios of MS to WPM were tested, including 7MW, a mixture of MS and WPM (7:3, v/v), 6MW (6:4), 5MW (=MW), 4MW (4:6), and 3MW (3:6). The survival rate, the length of the longest shoots, and the number of useable shoots were recorded 60 d after culture on each medium. Each treatment consisted of five flasks containing 20 segments, and these experiments were conducted four times. All multiplication media contained 0.8% (w/v) agar (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and 0.2% (v/v) Plant Preservative Mixture (PPMTM, Plant Cell Technology, DC).

The useable shoots cultured on MW medium with 20 μM zeatin were cut out of shoot clumps and planted in half-strength MW (1/2MW) supplemented with 0.2% (w/v) Gellan gum (Wako), but without plant growth regulators. Five to eight shoots were placed vertically in a 100-mL Erlenmeyer flask containing 40 mL medium. After 60 d culture, each rooted shoot was classified into 1 of 4 degrees of root system growth, from poor (score 1) to well-developed (score 4) (Suzuki et al., 1992). In each experiment, 120 shoots of ‘Kunisato 35 Gou’ and 50 shoots of No. 19 were used, and these experiments were conducted six times.

All media contained 2% (w/v) sucrose. The pH of each medium was adjusted to 4.8 with HCl before autoclaving.

Irrespective of rooting, all the shoots were planted in plastic pots (10 cm in diameter and 8 cm in depth) containing fine vermiculite (Asahi-Kogyo Inc., Okayama, Japan) and covered with transparent plastic film. In each pot, 12 to 15 plantlets were planted, watered, and fertilized occasionally with 0.1% Hyponex (N-P-K, 5-10-5; Hyponex Japan Corporation, Ltd., Osaka, Japan). To acclimatize the plantlets to ambient humidity, small holes were gradually pierced in the film, which was removed 50 d after potting. The rooting and survival of shoots were recorded 60 d after planting. In each experiment, 12 to 89 shoots were used, and these experiments were conducted six times.

After the completion of acclimatization, shoots with roots were planted singly in 57 × 57-mm Jiffy pots (Jiffy Preforma Production K.K., Yokohama, Japan) filled with one of five types of soil: vermiculite, Metro-Mix®360 (Sun Gro, Horticulture Distribution Inc., Washington DC, USA), Andosol, a mixture of Andosol and peat moss (1:1, v:v), or a mixture of Kanuma soil (volcanic tuff, 2 to 3 mm diameter) and peat moss (1:1, v:v). After trimming plantlets to 7 cm in height, they were put in the growth chamber, which was maintained at 25 °C under a 16-h photoperiod with a photon flux of 80 μmol･m-2･s-1 provided by cool white fluorescent lamps. These conditions were the same as those for the multiplication and rooting cultures. The plantlets were watered and fertilized with 0.1% Hyponex on a regular basis. The length of shoots was recorded 60 d after planting. In each experiment, four plantlets grown in each type of soil were used and these experiments were conducted six times.

In the middle of February, April, June, and August, the plants in the Jiffy pots were transplanted into the plastic pots (10 × 10 × 10 cm) containing the same soil and placed in a heated greenhouse in February and March and in an unheated greenhouse after April. The plants were watered and fertilized with 0.1% Hyponex on a regular basis. The length of shoots was recorded 60 d after transplanting. In each experiment, four plantlets grown in each type of soil were used. The pH of soil was periodically measured using a digital pH meter (DHP-1, Atago Co., Ltd., Tokyo, Japan).

All data, except for the degree of root system development, were subjected to one- or two-way (basal media or soil × clone) analysis of variance (ANOVA). The percentage data was subjected to arcsin transformation prior to ANOVA. However, there were significant interactions; therefore, the data for each clone were evaluated using a protected least significant difference (LSD) test. The effect of each clone on the degree of root system development was evaluated using the Kruskal-Wallis test.

**RESULTS**

The type of shoot segment used for micropropagation did not significantly affect the number of useable shoots or the length of the longest shoots of ‘Kunisato 35 Gou’ measured 60 d after culturing on MW medium supplemented with 20 μM zeatin (Table. 1). However, the 1-node segments occasionally produced no shoots. On the other hand, the longer the shoot segment from No. 19 that was used, the more useable shoots were produced and the longer the longest shoot grew (Table. 1). Shoot growth of ‘Kunisato 35 Gou’ was more vigorous than that of No. 19, although all the shoots of both clones survived on MW medium.

Almost all of the shoots, 95% or more, survived on each of the five types of basal media, except for ‘Kunisato 35 Gou’ grown on 3MW medium (85%), and No. 19 grown on 7MW medium (75%) (Table 2). Although there was no significant difference, the most useable shoots for either clone tended to be produced on 5MW (=MW) medium. The longest shoots of ‘Kunisato 35 Gou’ grown on 5MW and 6MW were longer than those grown on 3MW, while there was no trend for shoot length for No. 19. Regarding shoot growth, the basal medium used in this study did not alter the superiority of ‘Kunisato 35 Gou’ to No. 19.

Shoot growth did not correlate with rooting. Specifically, the rooting percentage of either clone was approximately 40% in the rooting medium, and there was no significant difference in the degree of root system development between 3.0 (‘Kunisato 35 Gou’) and 2.5 (No. 19). Moreover, more than 90% of the shoots which did not root in the rooting medium rooted 60 d after planting in vermiculite. During the acclimatization period, almost all of the shoots survived. More specifically, 97% of ‘Kunisato 35 Gou’ shoots with roots, 90% of ‘Kunisato 35 Gou’ without roots, 99% of No. 19 with roots, and 97% of No. 19 without roots had survived at the completion of acclimatization.

The soil used in Jiffy pots and plastic pots affected the growth of plantlets (Table 3 and Fig. 1). Both clones grew best in the mixture of Andosol and peat moss and in the mixture of Kanuma soil and peat moss when grown in the growth chamber and the greenhouse. The leaves of these plantlets were large and their stems were thick. At the end of the growing season, the growth increment varied with the length of growth period, but the plantlets grown in either the mixture of Andosol and peat moss or in the mixture Kanuma soil and peat moss still showed the best growth among plantlets transplanted at the same time. The plantlets growing in Metro-Mix®360 grew the most poorly; some leaves of the new shoots became light green and some had died by the end of growing season.

The pH of vermiculite, Metro-Mix®360, Andosol, the mixture of Andosol and peat moss, and the mixture of Kanuma soil and peat moss was originally 7.6, 6.2 6.1 4.9, and 4.3, respectively. Three months after transplanting, the pH of the mixture of Andosol and peat moss and the mixture of Kanuma soil and peat moss were still under 6.0, while the pH of the other soils was around 7.0. After four months, all soils remained around pH 7.0.

**DISCUSSION**

Generally, 1-node segments have been used for the multiplication of blueberry shoots (Tetsumura et al., 2008, Wolfe et al., 1996). However, some 1-node segments of ‘Kunisato 35 Gou’ and No. 19 did not grow on the MW medium. Therefore the optimal node number of segments used for shoot multiplication and elongation was investigated. The segment types of ‘Kunisato 35 Gou’ did not affect shoot growth. Thereafter, 2-node segments were used for subsequent experiments because they can be obtained more efficiently from the useable shoot than segments containing three or more nodes. However, the 5-node segments from No. 19 were the most productive for micropropagation. The multiplication rate of No. 19, however, was low; one 5-node segment, which was harvested from a useable shoot, produced only 1.2 useable shoots two months after it was cultured.

We previously found that MW medium produced better shoot growth than MS and WPM media for highbush blueberry cultivars (Tetsumura et al., 2008). The advantage of using MW medium had not yet been tested on rabbiteye blueberry, and an optimum mix ratio for MS and WP had not yet been investigated. The results of two rabbiteye blueberry clones tested here revealed that an equal mixture of MS and WP, designated MW, was the best basal medium. In addition to cultivated *Vaccinium*, wild *Vaccinium* has also been proven to be efficiently micropropagated on MW medium (Sato-Yamauchi et al., 2012).

Treatment with auxins is generally not required for rooting of blueberry microcuttings (Gonzalez et al., 2000, Isutsa et al., 1994, Meiners et al., 2007, Tetsumura et al., 2008). Therefore, we did not treat shoots of ‘Kunisato 35 Gou’ and No. 19 with auxins prior to planting in rooting medium without plant growth regulators. However, the rooting percentage was moderate. Subsequently, shoots with or without roots were planted in vermiculite, as some reports had indicated successful *ex vitro* rooting during blueberry micropropagation (Gonzalez et al., 2000, Isutsa et al., 1994, Sato-Yamauchi et al., 2012, Suzuki et al., 1992, Wolfe et al., 1983). This method eventually resulted in more than 90% rooting of the shoots that had already been acclimatized. Although it may be possible to simplify this method, it may be useful for increasing rooting percentages of blueberry microcuttings, particularly short ones, with otherwise moderate rooting ability (Wolfe et al., 1983).

Rabbiteye blueberry plants grow well in acidic soil, pH 4.3-5.0 (Eck, 1988). In this study, the admixture of peat moss made the pH of the potting soil below 5.0, which strongly promoted the growth of plantlets of these two clones. The mild acidity of the soil mixed with peat moss remained relatively stable for three months in the greenhouse. After four months, there were no differences in pH among the potting soils, but the plantlets planted in the soils mixed with peat moss continued to grow best. The soil pH must have a greater impact on the growth of smaller rabbiteye blueberry plantlets. Meanwhile, the plantlets in Metro-Mix®360 showed the worst growth, despite vermiculite and Andosol being neutral soils. The pH of the potting soil is evidently important for the growth of these two clones, while other soil characteristics are also important (Korcak, 1986).

‘Kunisato 35 Gou’ and No. 19 were bred for blueberry leaf tea production. Hence, the growth of *in vitro* shoots, and the growth of plantlets in plant factories,or in plantations are all important. The original ‘Kunisato 35 Gou’ clone showed more vigorous growth in the field than did the original No. 19 clone, so only the former was registered as a new cultivar. Superior *in vitro* shoot growth of ‘Kunisato 35 Gou’ was also observed in this study. Shoot multiplication of ‘Kunisato 35 Gou’ using a bioreactor (Debnath, 2009) might be a practical method for *in vitro* leaf tea production. On the other hand, No. 19 may be useful in plant factories, because its *in* and *ex vitro* rooting ability and post-acclimatization growth rate were the same as those of ‘Kunisato 35 Gou’. We have been investigating how to increase proanthocyanidin content in the leaves of plantlets in a plant factoryand in *in vitro* shoots (Fuse et al, 2010 and 2012).

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**Table 1. Effects of segment types on the number of useable shoots of at least 10 mm in length (Wolfe et al., 1983), and the length of the longest shoots of two rabbiteye blueberry clones, ‘Kunisato 35 Gou’ and No. 19.**

Segment type

Clone 1-node 2-node 3-node 4-node 5-node

Number of useable shoots

‘Kunisato 35 Gou’ 2.7 a 3.5 a 3.3 a 3.2 a 3.3 a

No. 19 0.4 c 0.6 bc 0.8 bc 1.0 ab 1.2 a

Length of the longest shoots (mm)

‘Kunisato 35 Gou’ 19.9 a 20.7 a 21.6 a 23.4 a 21.2 a

No. 19 13.0 c 13.9 c 16.0 ab 14.5 bc 16.7 a

Means were derived from 6 replicates of 20 segments each. Means in the same row followed by the same letter were not significantly different according to LSD test at *P* ≤ 0.05.

**Table 2. Effects of basal medium on survival rate, the number of useable shoots of at least 10 mm in length (Wolfe et al., 1983), and the length of the longest shoots of two rabbiteye blueberry clones, ‘Kunisato 35 Gou’ and No. 19.**

Basal medium

Clone 3MW 4MW 5MW 6MW 7MW

Survival (%)

‘Kunisato 35 Gou’ 85 a 95 a 95 a 95 a 100 a

No. 19 95 a 95 a 98 a 95 a 75 b

Number of useable shoots

‘Kunisato 35 Gou’ 2.7 a 2.7 a 4.0 a 3.9 a 3.6 a

No. 19 1.1 a 1.1 a 1.3 a 1.0 a 1.0 a

Length of the longest shoots (mm)

‘Kunisato 35 Gou’ 18.3 b 19.1 ab 21.6 a 21.8 a 21.0 ab

No. 19 15.6 a 14.4 a 16.2 a 15.7 a 16.6 a

Means were derived from 6 replicates of 20 segments each. Means in the same row followed by the same letter were not significantly different according to LSD test at *P* ≤ 0.05.

**Table 3. Effects of soil on the total shoot length (cm) of plantlets of two rabbiteye blueberry clones, ‘Kunisato 35 Gou’ and No. 19.**

Soil type

Days after Andosol and Kanuma soil and

Clone planting Vermiculite Metro-Mix®360 Andosol peat moss peat moss

‘Kunisato 35 Gou’ 60 24.1 b 18.7 b 23.1 b 34.6 a 37.5 a

120 37.9 bc 20.9 c 51.5 b 83.3 a 89.6 a

No. 19 60 28.0 ab 21.8 b 28.2 ab 39.8 a 40.0 a

120 37.3 b 28.7 b 54.5 b 102.7 a 100.5 a

Plantlets were cut to 7-cm sections when planted in Jiffy pots and transplanted into the same soil in plastic pots 60 d after growth in Jiffy pots. Means were derived from 6 replicates (60 d) and 4 replicates (120 d) of 4 plantlets each. Means in the same row followed by the same letter were not significantly different according to LSD test at *P* ≤ 0.05.

Figure captions

Fig. 1 Plantlets of ‘Kunisato 35 Gou’ and No. 19 planted in Jiffy pots filled with vermiculite (1), Metro-Mix®360 (2), Andosol (3), a mixture of Andosol and peat moss (4), or a mixture of Kanuma soil and peat moss (5) 60 d after planting. Bars: 20 cm.