Efficient In Vitro Screening for Higher Soil pH Adaptability of Intersectional Hybrids in Blueberry

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Abstract. We tested efficient in vitro methods for screening the genotypes with higher pH tolerance using multiple shoots of intersectional hybrids between *Vaccinium corymbosum* ‘Spartan’ and *V. bracteatum*. The response of the four hybrid clones tested to different pH levels was clone-dependent in vitro. An apparent difference was found in the rooting rate among the hybrid clones even at higher pH levels; the rooting rates of JM4 (91%) at pH 8.0 indicated a significantly high value compared with other clones (JM1: 24%, JM2: 9%, JM3: 8%, ‘Spartan’: 0%). Furthermore, JM4 showed constantly high rooting rates (91% to 100%) at all pH levels with no significant differences. Similar differences in the root characters of the hybrids were also confirmed by checking the viability of roots using fluorescein diacetate (FDA)/propidium iodide (PI) staining after dipping the roots of in vitro-produced shoots in liquid medium at different pH levels for 6 hours. These results suggest that an in vitro screening method using the rooting rate of multiple shoots and the viability test of roots by FDA/PI staining as a marker could become a very useful tool for the selection of germplasm with tolerance to higher pH within a short time using small planting spaces. In addition, JM4, which showed a high rooting rate at pH 8.0, could be useful in breeding new cultivars with higher pH tolerance.

The plants of the genus *Vaccinium* are “acid-loving” and generally require soils with pH below 5.8 for achieving high vigor (Hancock et al., 2008). Therefore, higher pH is one of the most important factors among the abiotic stresses limiting the growth of blueberries (Chandler et al., 1985). The soil pH of many orchards in Japan is 5.5 or higher as a result of the use of fertilizers with calcium-containing chemicals. Consequently, the growth of blueberries planted in the areas with high soil pH is restricted (Suzuki et al., 1999). Most blueberry breeders have not

focused on higher pH adaptation, although useful genetic variation suitable for the cultivation at higher pH levels may exist in several wild species (Rowland et al., 2011).

In Japan, 19 native species of the genus *Vaccinium* are distributed from Hokkaido to the Kyushu region (Yamazaki, 1989). The evergreen shrub, shashanbo (*V. bracteatum* section *Bracteata*), is rather common and distributed from the east to the west of Japan (Tsuda et al., 2013). This shrub species is of interest to blueberry breeders because it has a wide and deep root system, which makes the plants drought-tolerant, and it can grow well in higher pH soils compared with the other species belonging to the section *Cyanococcus* (Karizumi, 1979; Kunitake et al., 2006; Luby et al., 1991). Therefore, we produced intersectional hybrids between colchicine-induced tetraploid shashanbo and the tetraploid highbush blueberry ‘Spartan’ (*V. corymbosum* section *Cyanococcus*) (Tsuda et al., 2013). These intersectional hybrids might be useful as a good germplasm source to breed highbush blueberries that are more

adaptable to a broad range of soil conditions, including higher pH.

Field and greenhouse screening procedures have mainly been used to identify blueberry individuals or populations more tolerant to upland soils (Korcak, 1986; Korcak et al., 1982) or higher pH (Finn et al., 1987, 1993a, 1993b). These procedures might be useful for successful selection but require wide space and high costs for maintaining the plants and controlling the environmental conditions. In contrast, an in vitro screening system can be precisely or sufficiently controlled to allow nutrient or pH level adjustments so that it provides a more highly controlled environment than field or most non-field screening methods (Finn et al., 1991). Moreover, blueberries are one of the few fruit crops that can be screened at a whole-plant level in vitro as a result of their small seedling size and relatively slow growth rate. Finn et al. (1991) reported that in vitro screening in concert with a traditional breeding program could be effective for improving blueberry tolerance to higher pH.

The objectives of the present study were 1) to examine the response of intersectional hybrids between shashanbo and highbush blueberry ‘Spartan’ to different pH levels; and 2) to establish an in vitro screening system using multiple shoots to identify individuals that exhibit higher pH tolerance in *Vaccinium*.

Materials and Methods

Four intersectional hybrids, JM1, JM2, JM3, and JM4, and their parents, shashanbo (VB4x-1 and VB4x-2: seed parent of hybrids) and the highbush blueberry ‘Spartan’ (pollen parent of hybrids) were used in the present study. The shoots were taken from adult plants growing in pots in a greenhouse at Kibana Agriculture Science Station, University of Miyazaki, Miyazaki, Japan. After the leaves were removed, the nodal segments were rinsed for 5 min in running tap water, surface-sterilized for 15 min in 5% sodium hypochlorite solution containing 100 µL·L⁻¹ Triton X-100 (Nacalai Tesque, Kyoto, Japan), and washed three times with sterile water. The axillary buds as explants were aseptically dissected from the nodal segments and each planted in a test tube (3.0 cm × 12.0 cm) containing 10 mL of MW medium [a mixture of equal parts of Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Woody plant medium (WPM) (Lloyd and McCown, 1980), a final concentration of MW medium is half of MS and WPM medium] (Tetsumura et al., 2008), which contained 5 mg·L⁻¹ zeatin [6-(4-hydroxy-3-methylbut-2-enylamino)purine] (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), 0.8% (w/v) agar (Wako), 2% (w/v) sucrose, and 0.2% (v/v) Plant Preservative Mixture (PPM™; Plant Cell Technology, Washington, DC). The pH of the medium was adjusted to 4.8 with HCl before autoclaving. After regeneration of the plantlets, the nodal segments were subcultured on MW medium at 2-month intervals over 1 year to induce and proliferate multiple shoots.

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For the *in vitro* pH test, five kinds of media with different pH levels were prepared using half-strength MW (1/2 MW) supplemented with 1.0% (w/v) agar (Wako) and 2% (w/v) sucrose but without plant growth regulators. Media with pH levels of 4.0, 5.0, 6.0, 7.0, and 8.0 were formulated by adjusting with filter-sterilized HCl or NaOH after autoclaving. In this experiment, we used shoots of only ‘Spartan’ and the hybrids (JM1, JM2, JM3, JM4) because shashanbo was difficult to proliferate shoots in *in vitro* culture. The *in vitro* shoots without shoot apices were cut into 20-mm long segments and five segments with four to five leaves and axillary buds were placed in a 300-mL plant box (60 × 60 × 100 mm; CUL-JAR300; Iwaki, Tokyo, Japan) containing 40 mL medium. For each pH treatment, three plant boxes with 15 shoots each were used and the experiment was repeated three times. After 8 weeks of culture, the survival rate of the plantlets, the length of the longest root, and the length of the longest shoots were recorded. We counted the root and shoot of 1 mm or more, and the shoot with minimum one root of 1 mm or more was regarded as a rooted shoot. During the culture period, the rooting rate was recorded every 2 weeks until 8 weeks.

We assessed the effect of the medium pH on the viability of the root apex tissue by FDA/PI staining (Jones and Senft, 1985). As the material used for this experiment, we selected rooted shoots among the shoots cultured for 8 weeks by the same method as the *in vitro* pH test. Three rooted shoots were transplanted to a 300-mL plant box containing 40 mL 1/2 MW liquid medium supplemented with 2% (w/v) sucrose but without plant growth regulators. Liquid media with pH of 4, 6, and 8 were formulated by filter-sterilized HCl or NaOH after autoclaving. Stock solutions of FDA and PI were prepared by dissolving 5 mg·mL⁻¹ in acetone and 40 µg·mL⁻¹ in water, respectively, and stored at 5 °C according to the preparation method of Suzuki et al. (1999) with some modifications. After 3 h and 6 h of culture in liquid medium, treated roots were dipped in the FDA/PI solution (5 µg·mL⁻¹ FDA, 12.5 µg·mL⁻¹ PI) for 5 min at room temperature. After staining, the roots were rinsed with distilled water and then the stained tips were cut and examined for the cell viability and root morphology under an optical microscope (Olympus BX51; Olympus Optical, Tokyo, Japan) at 40× magnification. At least three shoots with 10 root tips each were observed per treatment.

In the *in vitro* pH test, response to higher pH differed clearly in JM1 and JM4. Accordingly, we selected JM1 and JM4 for the soil pH test and ‘Spartan’ was used as a control. The shoots were propagated by the method described previously. The shoots were cut into 20-mm long segments and planted in cell trays (20 × 20 × 40 mm) containing a soil mixture of vermiculite and perlite (1:1). The cell trays were placed in Jiffy half trays (270 × 270 × 60 mm) with transparent domes (270 × 270 × 60 mm). The plantlets were watered from the base as 0.1% Hyponex solution

(5N-10P-5K; Hyponex Japan Corp., Osaka, Japan) (Tetsumura et al., 2012), the pH of which was adjusted to 4.0 with H₂SO₄ or 8.0 with NaOH, and the watering solutions were replaced every 2 d. We used three replicates of 11 shoots for each treatment. The survival rate of plantlets, the rooting rate, the length of the longest root, and the length of the longest shoot were recorded 6 weeks after the culture.

All cultures were maintained at 25 °C under a 16-h photoperiod with a photon flux of 60 µmol·m⁻²·s⁻¹ provided by cool white fluorescent lamps. The results were evaluated using Tukey’s multiple range test or *t* test.

Results and Discussion

When the shoot segments excised from *in vitro* multiple shoots were cultured on media with different pH levels, the shoot explants of intersectional hybrids between shashanbo and highbush blueberry ‘Spartan’ showed a varied response to different pH levels of culture medium (Table 1; Fig. 1). Although all of the shoots of each hybrid clone and ‘Spartan’ survived on the media at pH 4.0 to 7.0 after 8 weeks of culture, survival rates at pH 8.0 showed a significant difference between hybrids (JM1: 100%, JM2: 96%, JM3: 84%, JM4: 100%) and ‘Spartan’ (24%). Root formation mostly initiated after 2 weeks of culture but delayed with the increase of medium pH (Fig. 2). In all hybrid clones and ‘Spartan’, rooting rates attained to almost maximum values at 4 weeks of culture except for JM2, which showed an increase until 8 weeks. The rooting rate of hybrids decreased with increase in pH level. An apparent difference was found in the rooting rate among the hybrid clones even at higher pH levels; the rooting rates of JM4 (91%) at pH 8.0 indicated a significantly high value compared with other

clones (JM1: 24%, JM2: 9%, JM3: 8%, ‘Spartan’: 0%). Furthermore, JM4 showed constantly high rooting rates (91% to 100%) at all pH levels with no significant differences. The length of the longest root of JM1, JM2, and JM3 significantly became shorter with an increase in pH level (Table 1). Although no apparent effect of pH on the length of the longest root was observed in ‘Spartan’, JM4 showed a constantly high length of the longest root (37.8 to 47.2 mm) at all pH levels with no significant differences. The longest root of JM1 and JM4 at pH 5.0 and 6.0 were significantly longer than other clones. In addition, JM4 at pH 7.0 and 8.0 had a significantly high length of the longest root compared with other clones. In contrast, the difference in pH levels did not affect the length of the longest shoot in each of JM2, JM3, JM4, and ‘Spartan’ (Table 1). In JM1, however, the maximum shoot length at lower pH was significantly longer than that at higher pH. Furthermore, JM4 at pH 7.0 and pH 8.0 had a significantly high length of longest shoot compared with other clones, similar to the longest root.

To assess the direct effect of pH on the viability of roots, rooted shoots of JM1, JM4, and ‘Spartan’ were transferred to liquid media with three pH levels (pH 4.0, 6.0, and 8.0). After 3 and 6 h of incubation, the roots were stained with FDA/PI solution (Fig. 3). After 3 h of incubation, each clone showed clear staining with both dyes in all of the roots, and no differences in viability or morphological characteristics were observed among the treated pH and clones, indicating that they remained viable at all pH levels (data not shown). Although the roots of ‘Spartan’ and JM1 were damaged in all treatments at 6 h, the roots of JM4 had no damage at all pH levels. These results suggest that JM4 has

Table 1. Effect of medium pH on survival rate, the length of the longest root, and the length of the longest shoot in intersectional hybrids (JM1 to 4) and ‘Spartan’.^z

Clones	Medium pH									
	4.0		5.0		6.0		7.0		8.0	
	<i>Survival rate (%)</i>									
JM1	100 ns ^y	NS ^x	100	NS	100	NS	100	NS	100	x
JM2	100 ns		100		100		100		96	x
JM3	100 ns		100		100		100		84	x
JM4	100 ns		100		100		100		100	x
Spartan	100 a		100 a		100 a		100 a		24 b	y
	<i>Length of the longest root (mm)</i>									
JM1	43.8 a ^y	x ^x	45.2 a	x	43.2 a	x	14.4 b	y	5.4 b	y
JM2	2.6 b	z	2.4 b	y	1.7 b	y	8.8 a	y	1.3 b	y
JM3	27.2 a	y	9.9 ab	y	8.1 b	y	8.7 b	y	1.0 b	y
JM4	47.1 ns	x	43.2	x	47.2	x	43.4	x	37.8	x
Spartan	2.9 ns	z	11.5	y	4.6	y	13.1	y	0	y
	<i>Length of the longest shoot (mm)</i>									
JM1	16.4 a ^y	xy ^x	18.9 a	x	18.9 a	xy	15.1 b	y	13.2 b	y
JM2	8.7 ns	z	11.1	y	9.4	z	12.9	y	8.9	y
JM3	15.8 ns	xy	10.5	y	10.6	z	13.0	y	11.6	y
JM4	19.8 ns	x	19.7	x	20.7	x	22.1	x	21.2	x
Spartan	13.8 ns	y	13.2	xy	12.4	yz	13.0	y	10.5	y

^zObservation was conducted after 8 weeks of culture.

^xDifferent letters, a–b, represent significant differences among different pH levels within the same clones in Tukey’s multiple range test at 5% level and ns shows nonsignificant difference.

^yDifferent letters, x–y, represent significant differences among different clones within the same pH level in Tukey’s multiple range test at 5% level and ns show nonsignificant difference.

tolerance to a broad range of pH levels in this method and that JM1 has less adaptability of the roots to the change of lower pH although slightly higher than 'Spartan'. The observation of root tissues by staining with FDA and PI is a simple technique that allows visible discrimination between viable and injured cells in *Vaccinium* (Suzuki et al., 1999). The present study revealed that FDA/PI staining is a useful tool to detect the genotypes with higher pH tolerance. Further studies are needed to clarify the mechanism involved in the genotypic difference in higher pH tolerance. In *Vaccinium*, it was confirmed that a higher pH had negative effects on seed germination and vigor and dry weight of seedlings (Finn et al., 1991) and shoot growth

(Wolfe et al., 1986) in vitro. Although the information on the effect of culture medium pH on rooting and root proliferation in *Vaccinium* is sparse (Meiners et al., 2007), our present findings revealed that the tolerance of roots to the higher pH levels could be evaluated by an in vitro culture method through the use of intersectional hybrids.

We next examined the tolerance of the JM1, JM4, and 'Spartan' to the higher pH in soil through the rooting test of in vitro-produced shoots (Table 2). Although survival rate of JM1 and JM4 was not influenced by pH level, the survival rate of 'Spartan' at pH 8.0 was significantly lower than that at pH 4.0. At pH 4.0, all clones examined showed

Fig. 1. Effect of medium pH on plant growth of two intersectional hybrids (JM1 and JM4) and 'Spartan'. Observation was conducted at 8 weeks after culture of in vitro shoots of 20 mm long. Bar = 5 cm.

Fig. 3. Effect of medium pH on root viability of two intersectional hybrids (JM1 and JM4) and 'Spartan'. Roots of the in vitro plantlets were soaked in liquid media with different pH levels for 6 h. Then roots were stained with fluorescein diacetate/propidium iodide solution. Green and yellow cells were evaluated as viable, whereas red cells were scored as damaged. Bar = 500 µm.

Fig. 2. Effect of medium pH on rooting in four intersectional hybrids (JM1 to 4) and 'Spartan' after 8 weeks of culture. ²Different letters represent significant differences among different pH levels within the same period (weeks) in Tukey's multiple range test at 5% level.

Table 2. Effect of soil pH on survival rate, rooting rate, the length of the longest root, and the length of the longest shoot in intersectional hybrids (JM1 and JM4) and ‘Spartan’.^z

Clones	Soil pH		
	4.0	8.0	
		<i>Survival rate (%)</i>	
JM1	97 ns ^y	100 a	NS ^x
JM4	94	91 a	NS
Spartan	100	73 b	**
		<i>Rooting rate (%)</i>	
JM1	78 a	30 ab	*
JM4	91 a	69 a	NS
Spartan	24 b	0 b	—
		<i>Length of the longest root (mm)</i>	
JM1	22.3 a	6.0 b	**
JM4	20.4 a	14.6 a	NS
Spartan	2.2 b	0 b	—
		<i>Length of the longest shoot (mm)</i>	
JM1	15.5 a	9.6 a	**
JM4	14.6 a	8.6 a	*
Spartan	4.9 b	1.7 b	*

^zObservation was conducted after 6 weeks of culture.

^yDifferent letters, a–b, represent significant differences among clones within the same pH level in Tukey’s multiple range test at 5% level and ns shows nonsignificant difference.

^xAsterisks represent significant differences between pH 4.0 and 8.0 within the same clones in *t* test at **1% or *5% level and ns shows nonsignificant difference.

an almost 100% survival rate. At pH 8.0, however, the survival rate of ‘Spartan’ decreased to 73%, which was significantly low compared with the two hybrids. All clones showed low values of rooting rate at pH 8.0 compared with those at pH 4.0. However, the rooting rate of JM4 was not significantly different between pH 4.0 (91%) and pH 8.0 (69%). The rooting rate of hybrids was significantly higher than ‘Spartan’ at pH 4.0, but the rooting rate of JM1 and ‘Spartan’ at pH 8.0 was not significantly different. Differences across the clone and pH levels of the length of the longest root indicated similar tendency as rooting rate. In contrast, the maximum shoot length at pH 4.0 was significantly longer than that at pH 8.0 in all clones. Furthermore, the longest shoot of two hybrids was significantly longer than ‘Spartan’ in each pH level. These results confirmed that the soil pH test could generally give similar results to the in vitro test and that the pH level and genotype affect the rooting rates of intersectional hybrids of blueberry.

Blueberry researchers have tried to formulate an inclusive theory regarding blueberry intolerance to higher pH soils (Korcak, 1989). Although this research has allowed the development of cultural systems with suitable medium to the plant, it has not yet led to the development of cultivars adaptable to variable soil conditions (Finn et al., 1991). An in vitro screening method for identifying

higher pH tolerance of seed and seedlings was developed by Finn et al. (1991). However, seeds are usually not suitable for this screening method because normal seeds of blueberry do not germinate uniformly as a result of the immature nature (Suzuki et al., 1999). Therefore, to obtain stable results with good reproducibility, the use of multiple shoots for in vitro screening is useful and necessary. The advantage of this approach is that it enables the propagation of the selected superior clones in a short time by micropropagation, and field testing can be started quickly.

In conclusion, we have succeeded to produce blueberry clones with higher pH tolerance by intersectional hybridization between *V. corymbosum* and *V. bracteatum* and demonstrated an efficient in vitro method for screening the hybrid clones of *Vaccinium* with higher pH tolerance using multiple shoots combined with FDA/PI staining of roots. These results suggest that in vitro screening method using both the rooting rate of multiple shoot and the viability assessment of roots by FDA/PI staining could become a very useful tool for the selection of germplasm with tolerance to higher pH with very small planting spaces within a short time. Because the hybrid JM4 showed the potential for the adaptability against higher pH soil conditions, further investigations are necessary to confirm the actual growth of this clone at higher soil pH conditions to clarify the mechanism involved in the higher pH tolerance. Furthermore, the studies on flowering and fruiting habits and fruit quality in JM4 are important to use this clone for our breeding program.

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