Comparison of Growth Characteristics between Diploid and Colchicine – induced Tetraploid Grape Rootstocks

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Summary

Autotetraploids of three grape rootstocks, ‘Gloire de Montpellier’ (‘Gloire’, *Vitis riparia* Michx), ‘Rupestris St. George’ (‘St. George’, *V. rupestris* Scheele), and ‘Couderc 3309’ (‘3309’, *V. riparia* × *V. rupestris*), were induced by the colchicine treatment of micropropagated plantlets. Doubling of chromosomes in the selected colchicine-treated clones was confirmed by flow cytometric analysis of the immature leaves. The tetraploid leaves had larger stomata that were distributed at a lower density than those of the original diploid leaves. In the rooting culture, all tetraploids had much shorter root lengths than had the original diploids. Under rooting culture, tetraploid shoots of ‘Gloire’ and ‘St. George’ were shorter than those of diploids, but tetraploids of ‘3309’ were not. In the acclimating stage in this experiment, the tetraploids, also had shorter shoots, internodes and roots than had the original diploid plants. In the greenhouse experiment, shoot of tetraploid rootstocks, likewise, made poorer growth than did those of the diploids, but they tended to have thicker stem diameters and the specific leaf weights (leaf dry weight/ leaf area). The thicker and shorter roots of the tetraploid rootstocks made their root system more compact than the diploids.

Key Words: flow cytometry, growth characteristics, grape rootstock, tetraploid, *Vitis* spp.

Introduction

Grape rootstocks commonly utilized in Japan do not satisfy all the demands for grape production. Most rootstocks had been selected for wine grapes (*V. vinifera* L.) under dry summers of the Europe, which may not be suitable for Japanese cultivars grown under a wet climate. In Japan, tetraploid table grapes of interspecific hybrids between *V. vinifera* and *V. × labruscana* Bailey are planted in about 40% of the total grape production area (total 20500 ha; 6620 ha for ‘Kyoho’ and 1370 ha for ‘Pione’) (MAFF Japan, 2000). These tetraploid grapes grafted onto common rootstocks (diploids), however, have some problems in flower fertility and berry coloring, which may be due to their vigorous shoot growth. On the other hands, Imai et al. (1987) reported that own-rooted ‘Kyoho’ and ‘Pione’ vines showed better berry setting than did the vines grafted onto ‘Teleki 8B’ rootstocks. This improvement of berry setting is thought to be related to the phenomenon that own-rooted tetraploids are relatively weak and compact, compared with the original diploid (Olmo, 1976;

Motosugi, 2000). Moreover, own-roots of scion cultivars have no tolerance to grape phylloxera (*Daktulosphaira vitifoliae* [Fitch.]) (Wapshere and Helm, 1987). Most grape production regions in Japan have been infested with phylloxera in the past. Thus, grapevines grown with their own-roots involve a risk by phylloxera infestation.

Many autotetraploid grapevines had been produced by colchicine treatment (Yamane and Kurihara, 1980; Thompson and Olmo, 1963). Nozuka et al. (2000) reported a very efficient method for the production of autotetraploid by *in vitro* treatment with colchicine and produced many tetraploid table grapes. However, there are no reports evaluating the growth characteristics of own-rooted tetraploids of grape rootstocks and their effects on the scion growth. In this study, we produced new tetraploids of three grapevine rootstocks by *in vitro* methods of Nozuka et al. (2000), and proved their ploidy by flow cytometric analysis, which has been recently demonstrated to be quick and efficient for ploidy determination of fruit trees (Ollitrault-Sammarcelli et al., 1994; Chalak and Legave, 1997; Costich et al., 1993; Tamura et al., 1995, 1996). Thereafter, the growth characteristics of the tetraploid rootstocks were compared with those of the original diploid rootstocks during *in vitro* rooting, acclimation stage, and growth in a greenhouse.

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Materials and Methods

Production of tetraploid rootstock by colchicine treatment in vitro

Shoot tips from three grape rootstocks; 'Gloire de Montpellier' ('Gloire', *V. riparia* Michx), 'Rupestris St. George' ('St. George', *V. rupestris* Scheele) and 'Couderc 3309' ('3309', *V. riparia* × *V. rupestris*) were sterilized with sodium hypochlorite solution (1% active chlorine) for 15 min, then rinsed in sterilized water. Shoot tips, which were dissected to 0.2–0.3 mm, were put into a liquid medium containing 1/3 strength of Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with 3% sucrose, 5 μ M 6-benzylaminopurine (BA) and 0.5 μ M 1-naphthaleneacetic acid (NAA). Shoots, derived from the shoot tips were transferred to MS medium gelled with 0.8% agar and supplemented with 3% sucrose, 5 μ M BA to proliferate shoots. Subsequent to shoot division, shoots were transferred to the shoot-elongation medium (MS + 2 μ M BA or 5 μ M Zeatin). One-node segments from the elongated shoot were incubated in 1/3 strength of MS liquid medium containing 0.01 or 0.1% colchicine for 3–7 days. Ten to thirty segments from each rootstock cultivar were treated, then the segments were rinsed in sterilized water, and their brown ends excised. Two to four weeks after transferring to shoot-proliferation medium, surviving segments with newly developed shoots were transferred to shoot-elongation medium. One month later, shoot tips (1–2 cm long) were cut from elongating shoots and transferred to a rooting medium consisting of 1/3N MS (MS with 1/3 nitrogen) containing 0.5 μ M NAA. Environmental conditions in *in vitro* culture and acclimation experiments were 50 μ mol $\cdot$ m⁻² $\cdot$ sec⁻¹ of light intensity under a 16-hr photoperiod, and 25 $\pm$ 1 $^{\circ}$ C.

Stomatal observation, flow cytometric analysis and chromosome counting

The plantlets, producing thicker roots than the original diploid shoot, were selected and proliferated in the rooting medium. The replicas of abaxial surface of the leaf from these selected clones were produced by the method after Hirose et al. (1992), using a cyanoacrylate instant adhesive. Stomatal density and guard cell lengths in the replica were measured with a digital microscope (VF2000, Keyence, Osaka).

For flow cytometric analysis, immature leaves of the selected clones grown *in vitro*, were collected and chopped with a razor blade in 1 ml of cold buffer consisting of 10 mM Tris-HCl (pH 7.0), 10 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, and 1% PVP K-30 according to Tamura et al. (1995) and Dickson et al. (1992). The chopped leaves in the buffer were chilled on ice for 5–10 min, stirred gently and the mixture then filtered through a 20 μ m meshed nylon filter. One-

tenth volume of propidium iodide solution (1 mg $\cdot$ ml⁻¹) was added to the filtrate and mixed. The mixture was incubated at room temperature for 5 min and analyzed with a flow cytometer (FACS Calibur, Becton-Dickinson, San Jose, CA). Chicken erythrocyte nuclei (CEN, Pinnacle Biosystems, Burlingame, CA) was utilized as a standard [2.33 pg DNA in a 2C nucleus (Johnston et al., 1999)]. The ploidy level of the selected plantlet was determined by comparing the relative fluorescence intensity with CEN.

For chromosome counting, root tips were pretreated in 0.002 M 8-quinolinol solution and fixed in Carnoy solution (ethanol: acetic acid, 3:1) and digested with 4% Cellulase Onozuka and 1% pectolyase Y-23 after Zhuang et al. (1990). The digested samples were tapped with tweezers in a drop of Carnoy solution to spread the macerated cells on a slide glass. After drying, the samples on the slide glass was stained with 2% Giemza's solution and observed under a light microscope.

Growth in rooting culture

One tetraploid clone of each rootstock was examined for growth characteristics. Five, leafless, one-node cuttings, obtained from micropropagated plantlets, were placed on 40 ml of rooting culture medium in a 200 ml glass bottle with a transparent plastic cap. Twenty to 25 cuttings from each diploid and tetraploid rootstock were cultured for 6 weeks, after which shoot length, number of nodes, total length of roots, average length of roots, and numbers of root per cutting were measured. For statistic analysis, data from the plantlets without roots were excluded.

Growth in acclimation stage

To examine the growth characteristics in acclimation stage, plants were transplanted into a polyethylene pot (9 cm diameter) filled with vermiculite and placed on the lighting shelf covered with transparent polyethylene film to maintain high humidity. Nutrient solution (N: P: K= 18.6: 1.7: 8.6 mM) was applied to each pot biweekly. After one month, shoot length, nodes per shoot, and mean length of the 3 longest framework roots were measured; the data for statistic analysis were collected from the 10 vines that showed superior growth in each rootstock.

Growth in glasshouse

The acclimated grapevines were transplanted into a larger polyethylene pot (18 cm diameter) filled with vermiculite: perlite (1:1) medium and transferred to a glasshouse kept above 18 $^{\circ}$ C. Due to high light intensity and low humidity, the vines transplanted to the greenhouse stopped growing for a few months, as if dormant. Therefore, all stems were headed back to about 5 nodes above the ground to stimulate sprouting. Five grams of coated, slow release (100 days) fertilizers (0.63 g N, 0.16 g P, 0.36 g K, 0.27 g Ca, and 0.04 g Mg) were

scattered on the pot surface. Water was supplied automatically twice a day. When the vines restarted their growth, all shoots, except the uppermost one on the stem, were removed for the growth character measurement. One month later, the length and weights (fresh and dry) of both shoot and root were measured. Internode length was measured for 3 internodes in the mid-portion of the shoot of each vine. Stem diameter was measured on the second or third internode from the base. The length of the 5 longest framework roots and thickness at 20 cm from the root tip were also measured. Root volume was calculated from the difference between the weight of the whole plant and the weight of the root portion submerged in water. The data for statistic

analysis were collected from the superior 10 vines in each tetraploid and the original rootstock cultivar.

Statistics

Because the experimental periods differed among every pair of diploids and tetraploids of each rootstock, two-factor (rootstock $\times$ ploidy) analysis of variance could not be used. Therefore, mean differences were analyzed by *t*-test between diploid and tetraploid of each rootstock. Percentage values were subjected to arcsine transformation before statistical analyses.

Results

Flow cytometry, chromosome counting and stomatal observation

Immature leaves from several plantlets having thick roots and large stomata, which were selected from each rootstock treated with colchicine and proliferated in rooting medium, exhibited typical histograms in flow cytometric analyses (Fig. 1). The left end peaks at about 100 in relative fluorescent intensity were caused by debris. Major peaks at around 200 in diploids or 380 in tetraploids represent the nuclei in the G_0 and G_1 phase in cell cycles; the minor peaks sometimes found at around double intensity of the major peak, for example, a peak in 'St. George' at 380, represents the nuclei in the G_2 phase. The relative fluorescence intensity of the peaks fluctuated somewhat in every replication. The mean values of G_1 and G_0 peaks among replications were compared between the selected clones, treated with colchicine, and the original diploid rootstocks. Flow cytometric analysis indicated that the selected clones of 'Gloire', '3309', and 'St. George' had almost twice as much nuclear DNA in G_0 and G_1 phase as the original diploid rootstocks. Chromosome counts on root tips also confirmed that these clones had around 76 chromosomes, which was twice those of the original diploid rootstocks ($2n=38$).

The guard cells in the leaves of tetraploids, derived

Fig. 1. Flow cytometric analysis of nuclear DNA from leaves of diploid and tetraploid grape rootstocks. Arrows show the relative fluorescence intensity of 2C nuclei of CEN (Chicken erythrocyte nuclei, 2.33 pg DNA in a 2C nucleus).

Table 1. Length and density of stomatal guard cells on the abaxial leaf surface of diploid and tetraploid grape rootstocks grown *in vitro*.

Grapevines	Length of guard cell (μm)	Density of stomata (stomata $\cdot \text{mm}^{-2}$)
Gloire	38.6*** ^z	141.1***
Gloire (4x) ^y	49.9	90.9
3309	37.2***	188.3***
3309 (4x)	58.2	96.0
St. George	35.0***	144.2***
St. George (4x)	48.4	71.8

^z Mean difference was significant ($P < 0.001$) between the diploid and tetraploid in each rootstock cultivar.

^y (4x); tetraploid.

Table 2. Growth characteristics of diploid and tetraploid grape rootstocks in rooting culture.

Grapevines	Shoot length (cm)	No. of nodes	Internode length (cm)	No. of roots	Total root length (cm)	Average root length (cm)
Gloire	6.4*** ^z	3.7*	1.8*	5.1NS	24.4*	4.8***
Gloire (4x) ^y	3.6	3.0	1.2	4.9	13.5	2.7
3309	3.4NS	3.1NS	1.1NS	3.1NS	12.3NS	2.6*
3309 (4x)	3.0	3.1	1.1	3.1	9.7	2.2
St. George	9.8***	7.6*	1.3**	4.6NS	44.0**	9.8**
St. George (4x)	5.8	5.8	0.9	4.6	19.9	4.2

^z Mean difference between the diploid and tetraploid of each rootstock was significant (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$), or not significant (NS) ($n = 16 - 21$).

^y (4x); tetraploid.

Table 3. Growth characteristics of diploid and tetraploid of grape rootstocks in the acclimation stage

Grapevines	Shoot length (cm)	Number of nodes	Internode length (cm)	Average root length ^z (cm)
Gloire	25.6*** ^y	8.2NS	3.1***	22.4***
Gloire (4x) ^x	14.2	9.0	1.5	8.1
3309	27.0***	11.4**	2.3**	16.7**
3309 (4x)	18.2	9.2	2.0	11.4
St. George	37.1***	12.4***	3.0**	24.1***
St. George (4x)	19.4	8.7	2.2	7.7

^z Average of the 3 longest framework roots.

^y Mean difference between the diploid and tetraploid of each rootstock was significant (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$), or not significant (NS) ($n = 10$).

^x (4x); tetraploid.

from each rootstock, were 1.3–1.5 times longer and of lower densities than those of the original diploids (Table 1).

Growth in rooting culture

In the rooting culture, the shoot length, node number, and internode length in 'Gloire' (4x) and 'St. George' (4x) were significantly smaller than those of the original diploids (Table 2) but not those of tetraploid of '3309'. The frequency of roots did not differ between the diploids and tetraploids in all varieties. The total root length and average root length in 'Gloire' (4x) and 'St. George' (4x) were much shorter than those in the original diploids. Although the total root length did not differ significantly, the average root length of '3309' (4x) was statistically shorter than that of '3309'.

Growth in acclimation stage

During the acclimation period, the shoot lengths of all tetraploids were about 40% shorter than the original diploid rootstocks (Table 3), because their internodes were significantly shorter. The average length of the three longest framework roots in each vine was significantly shorter in all three tetraploids than those in the diploid rootstocks.

Growth in greenhouse

In greenhouse experiments, the shoot length of each tetraploid was reduced to about 40% ('Gloire' (4x)), 42% ('3309' (4x)), and 61% ('St. George' (4x)) of that of the original diploid, respectively (Table 4). The tetraploids had significantly fewer nodes/shoot and shorter internodes but somewhat larger stem diameters than the original diploids (Table 4, Fig. 2). The roots of tetraploid were significantly shorter but of much larger diameter than those of diploids (Table 5, Fig. 2); their volumes of the whole root system were, likewise, slightly smaller than those of the original rootstock but the difference was not significant. The stem dry weights of the tetraploids were significantly lighter, but not significantly so, except for '3309' (Table 5). Leaf characteristics were somewhat different between diploid and tetraploid rootstocks, but the specific leaf weight of tetraploid rootstock was significantly heavier (Table 6) because their leaves were thicker.

Discussion

Increasing ploidy generally results in larger cells, hence thicker and wider leaves, larger flowers and fruits, and thicker stems with shortened nodes. The size of stomata or guard cells indicates the ploidy level of

epidermal cells (Sanford, 1983) so we first selected colchiploids by their leaf shape, internode length, and root tip shape, and secondly, by microscopic observation of stomatal size, and finally, by flow cytometric analysis. The colchiploid, having large stomata, may be a periclinal chimera that consisted of tetraploid and diploid cell layers. If the colchiploid were a periclinal chimera, two major peaks would appear in the histogram in flow cytometric analysis, namely, the first peak may be the DNA from diploid cell layers, and the second peak may be from tetraploid cell layers of the periclinal chimera. In this experiment, however, the single major peak that appeared at a double fluorescent intensity of the G_0 and G_1 peak in the original diploid demonstrates that these colchiploids are tetraploids in all cell layers.

Decreased growth rate or reduced plant height with increased polyploidy of fruit trees have been observed as autotetraploid of grapevines (Motosugi, 2000), chromosome doubled persimmon, produced by colchicine treatment of protoplasts from 'Jiro' (Tamura et al., 1996), and nonaploid (9x) of kiwifruit (normal cultivars

Fig. 2. Photographs of 'Gloire' and 'Gloire' (4x) grown in a greenhouse. Horizontal bars show 100 cm.

Table 4. Shoot growth of diploid and tetraploid grape rootstocks grown in a greenhouse.

Grapevines	Shoot length (cm)	Number of nodes	Internode length (cm)	Stem diameter (mm)	Stem dry weight (g)
Gloire	136.8*** ^z	16.1**	13.9***	3.2NS	2.76**
Gloire (4x) ^y	54.0	11.0	7.2	3.3	1.12
3309	131.4***	18.8**	7.1***	3.0NS	1.32**
3309 (4x)	55.3	12.3	4.5	3.1	0.72
St. George	101.9**	25.1**	7.0**	2.3**	1.39*
St. George (4x)	62.5	19.6	5.9	2.6	0.86

^z Mean difference between diploid and tetraploid of each rootstock was significant (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$), or not significant (NS) ($n = 10$).

^y (4x); tetraploid.

Table 5. Root growth of diploid and tetraploid grape rootstocks grown in a greenhouse.

Rootstocks	Whole root volume (cm ³)	Average length ^z (cm)	Thickness ^y (mm)	Root dry weight (g)
Gloire	16.2NS ^x	69.8***	0.69***	2.73NS
Gloire (4x) ^w	13.9	31.7	1.55	2.03
3309	17.3NS	75.9***	0.42***	3.59*
3309 (4x)	15.9	38.4	1.03	2.46
St. George	12.2NS	70.1***	0.55***	1.67NS
St. George (4x)	10.0	35.2	0.95	1.15

^z Root length was measured on the 5 longest framework roots per vine.

^y Root thickness on the 5 longest roots per vine was measured at the point of 20 cm apart from root tip.

^x Mean difference between diploid and tetraploid of each rootstock was significant (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$), or not significant (NS) ($n = 10$).

^w (4x); tetraploid.

Table 6. Leaf characteristics of diploid and tetraploid grape rootstocks grown in a greenhouse.

Rootstocks	DW per leaf (mg)	Water Content (%)	Area per leaf (cm ²)	Specific leaf weight (mgDW · cm ⁻²)
Gloire	301.3** ^z	78.8NS	123.5NS	2.45***
Gloire (4x) ^y	390.9	78.9	118.3	3.31
3309	85.1NS	81.8NS	33.8**	2.58***
3309 (4x)	81.7	81.2	23.6	3.59
St. George	93.9***	81.7***	34.8***	2.69**
St. George (4x)	130.9	84.2	45.8	2.85

^z Mean difference between the diploid and tetraploid of each rootstock was significant (*; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$), or not significant (NS) (n=20).

^y (4x); tetraploid.

were hexaploid (6x)) (Ollitrault-Sammarcelli et al., 1994). The reduced vigor with increased polyploidy can be used for the production of dwarfing rootstock. Lee (1988) reported that the autotetraploids of commercial citrus rootstocks reduced the scion size. Grosser and Chandler (2000) also reported that a somatic hybrid (allotetraploid) between sour orange and Palestine sweet lime reduced the scion size of sweet orange. Beakbane (1967) reported a tetraploid chimera of M.13 apple rootstock that was about as dwarfing as M.9 but had structurally stronger roots. A few tetraploids of *Vitis* species related to grape rootstocks have been reported in some studies (Staudt, 1995; Tompson and Olmo, 1963), but none evaluated the growth characteristics of the tetraploids of grapevine rootstocks.

Our tetraploids rootstocks, 'Gloire' (4x), '3309' (4x) and 'St. George' (4x) have significantly shorter shoots, internodes, and roots than the original diploid rootstocks during rooting, acclimating, and greenhouse. Our other tetraploids derived from 'Kober 5BB' and 'Selection Oppenheim No.4 ('SO4')' (*V. berlandieri* Planch. × *V. riparia*), which have been acclimated in the greenhouse, also have shorter shoots and more compact root systems than the original diploids (data not shown). Eissenstat (1991) suggested that specific root length (SRL, root length / root dry weight) is an important factor for water and nutrient absorption because their uptake become more efficient. Although the actual SRL cannot be calculated without the measurement of total root length, tentative SRL values were calculated from the ratio of mean length of the five longest roots to the dry weight of the whole root in the greenhouse experiment (Table 5). The tentative SRL values (cm · g⁻¹ DW) of tetraploids which were: 19.1 in 'Gloire' (4x), 16.4 in '3309' (4x), and 34.7 in 'St. George' (4x) are significantly lower than those of the original diploids: 30.0 in 'Gloire', 23.7 in '3309', and 50.1 in 'St. George'. Therefore, tetraploid rootstocks may have a lower capacity for water and nutrient uptake. Root volume and dry weight in tetraploids were relatively smaller than those of diploids (Table 5), although the differences are not significant except for '3309' (4x). This difference in root growth

between tetraploid and diploid may diverge or converge during a long-term culture in the vineyard, so that further study on the growth and morphology of roots, especially SRL, and water and nutrient uptake of tetraploid rootstocks is needed.

In conclusion, autotetraploids of grapevines generally have coarse, compact root systems, compared with the original diploids so that their root systems may restrict the distribution of their roots in vineyard and reduce the growth of the scion grafted onto them. In a high-density planting system, flower shatter on tetraploid table grapes such as 'Kyoho' and 'Pione' was reduced by restricting root growth in a small volume (120 liter) (Imai et al., 1987). Thus, flower fertility of scion cultivars may be improved by the tetraploid rootstocks by limiting root growth. We demonstrated that young 'Kyoho' vines micrografted onto these three rootstocks, 'Gloire' (4x), '3309' (4x) and 'St. George' (4x), had reduced vegetative growth, compared to vines grafted onto the original diploid rootstocks (Motosugi et al., 1999).

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2倍体およびコルヒチン処理により作出した4倍体ブドウ台木における生育特性の比較

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摘 要

組織培養により増殖したブドウ台木 'Gloire de Montpellier' ('Gloire', *Vitis riparia* Michx), 'Rupestris St. George' ('St. George', *V. rupestris* Scheele) および 'Couderc 3309' ('3309', *V. riparia* × *V. rupestris*) においてコルヒチンによる染色体倍加処理を行った。コルヒチン処理個体より選抜した系統の幼葉についてフローサイトメトリーによる染色体倍加の確認を行った。得られた4倍体の葉においては元の2倍体台木と比較して気孔が大きく、その分布密度が低くなった。発根培養時において、3種類の台木の4倍体の根はすべても

との2倍体台木より短くなった。シュート長においては 'Gloire' および 'St. George' の4倍体でもとの2倍体より小さくなったが、'3309' では倍数性による差異がなかった。馴化期間において4倍体台木の新梢、節間および根の長さは元の2倍体台木より短くなった。ガラス温室に搬出後の生育においても4倍体台木では元の2倍体に比べ新梢生長は顕著に小さくなったが、茎径および比葉重は大きくなる傾向が認められた。4倍体台木では太く短い根をもつため、元の2倍体台木と比較して非常にコンパクトな形態の根系となった。