Evaluation of Seedlings from 11 *Citrus* Accessions for *In Vivo* Micrografting

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The very young seedlings of trifoliate orange, and its hybrids with trifoliate leaves as a marker, are usually used as a rootstock for *in vitro* and *in vivo* micrografting of citrus to eliminate viruses in spring in temperate regions. In tropical and subtropical regions or in summer, however, the production and use of trifoliate orange seedlings is difficult. Therefore, it is necessary to establish suitable micrografting using efficient *Citrus* seedlings adapted to these regions and summer. In decapitated seedlings for micrografting, adventitious shoots on the cut end of the epicotyls and shoots from cotyledon axillary buds are often formed. Hence, the potential for adventitious shoot formation and cotyledon axillary shoot formation was firstly studied with decapitated seedlings from 11 *Citrus* accessions and one *Poncirus* accession as a control. Mature seeds of the 12 accessions were germinated *in vivo* and seedlings of various ages (2-, 4-, and 8-week-old, 4-month-old and 8-month-old after germination) were decapitated at three (lower, middle, and upper) positions on the epicotyls. Adventitious shoot formation decreased with increases in the age of seedlings decapitated at eight weeks or 4 months after germination. The percentage of decapitated seedlings forming adventitious shoots was different in different accessions ranging from 0% to 100%, and increased with increases in decapitation height in the epicotyls. It was estimated from these results that 2-week-old seedlings of Natsudaidai, Shiikuwasha, ‘Hirado-buntan’, ‘Variegated Daidai’, and trifoliate orange ‘Flying Dragon’ had higher potential to support the initial growth of adventitious shoots, and cotyledon axillary shoots than the others, and that decapitation at the upper one-third and middle of epicotyls resulted in higher adventitious shoot formation than the lower one-third. In *in vivo* micrografting of satsuma mandarin on the seedlings of these accessions, Natsudaidai, ‘Hirado-buntan’, and ‘Variegated Daidai’ seedlings resulted in high micrografting success rates, whereas ‘Kabusu’, ‘Flying Dragon’ and Shiikuwasha seedlings resulted in very low success rates. The success rate decreased with increases in seedling age. It has become clear from these results that there is no relationship between the potential for shoot formation and micrografting success and that the 2-week-old seedlings of Natsudaidai, ‘Hirado-buntan’ and ‘Variegated Daidai’ are efficient rootstocks for micrografting.

Key Words: albino seedling, epicotyl, shoot tip, summer, tropical region.

Introduction

Citrus is widely cultivated from tropical to temperate areas and is one of the most important fruit tree types, with high economic value as a source of human nutrition worldwide. Some of diseases and harmful insects, however, result in low fruit quality and productivity. Fungicides and pesticides are in general useful for such kinds of infection, but it is very difficult to control virus

and/or virus-like pathogens with these chemicals. Shoot tip grafting or micrografting techniques are especially important to establish virus-free plants for perennial species such as fruit trees with difficult to culture shoot apices (Hussain et al., 2014). It was reported that in *Citrus*, virus and virus-like pathogens could be eliminated by micrografting (Abbas et al., 2008; Chand et al., 2013; Navarro et al., 1975; Sanabam et al., 2015; Singh et al., 2008). Juarez et al. (2015) reported that *in vitro* micrografting is also a very useful technique for (1) regeneration of somatic hybrids from difficult to germinate embryos, (2) regeneration of plants from irradiated shoots, (3) regeneration of plants from very difficult to germinate haploid embryos, (4) production of stable tetraploid plants of non-apomictic genotypes, and

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(5) regeneration of transgenic plants from very difficult to root shoots *in vitro*. In these cases, seeds of rootstock cultivars such as citranges with trifoliate leaves as a rootstock marker were used and the seedlings one to two weeks after germination were used as rootstocks for *in vitro* micrografting. Volk et al. (2012) carried out cryopreservation of citrus shoot tips using *in vitro* micrografting for recovery. ‘Carrizo’ citrange seeds were germinated in darkness for up to six weeks until used as a rootstock for micrografting. To enhance the scion growth in *in vitro* micrografting of ‘Washington Navel’ orange on ‘Troyer’ citrange seedlings, micrografting scions with two leaves were excised six weeks after micrografting and regrafted on 9- to 10-month-old seedlings of seven *Citrus* species, and the results suggested sour orange seedlings should be selected for rapid growth of scions obtained by micrografting (Sertkaya, 2004). In almost all of these *in vitro* micrografting studies, approximately 2-week-old etiolated seedlings of rootstock cultivars such as trifoliate orange, citranges and rough lemon were often used in initial *in vitro* micrografting and regrafted on other older vigorous seedlings 4–6 weeks after micrografting to enhance growth (Chand et al., 2013; Juarez et al., 2015).

In vivo micrografting was demonstrated by Takahara et al. (1986) who used 12- to 14-day-old trifoliate orange seedlings after germination as a rootstock for micrografting of several *Citrus* cultivars and obtained virus-free plants; their shoot apices were 0.2–0.4 mm and were 0.8–1.0 mm in length in all cultivars in combination with scion thermotherapy treatment. Thirty to 40 days after micrografting, the rootstock trifoliate orange seedlings with scions and expanded leaves were inarched on 2-year-old trifoliate orange seedlings to facilitate scion growth (Takahara et al., 1986). Oiyama and Kobayashi (1993) successfully produced vigorous plants of haploid clementine seedlings that showed very poor growth after germination through *in vivo* micrografting on trifoliate orange seedlings. After these reports, many *in vivo* micrografting studies were carried out to recover virus-free cultivars with some modification of the method (Nakajima et al., 2017; Ohta et al., 2011). In almost all of these *in vivo* micrografting studies, approximately 2-week-old trifoliate orange seedlings were used in the initial *in vivo* micrografting and regrafted on other aged vigorous seedlings 4–6 weeks after micrografting to enhance growth.

Since *Citrus* seedlings tend to produce adventitious shoots on the decapitated end of epicotyls (Chand et al., 2013), trifoliate orange and its hybrids were used for micrografting to identify adventitious shoots from the rootstocks (Murashige et al., 1972; Navarro et al., 1975). Trifoliate orange, known to be an exclusive rootstock for satsuma mandarin in Japan, exhibits dormancy, i.e., the buds of this deciduous trees sprout after 1000 h exposure to a chilling temperature lower than 7°C (Takishita, 2016) and the mature seeds also nor-

mally germinate after encountering chilling temperature in autumn and winter. This makes it difficult to use the seedlings for micrografting in tropical and subtropical areas or in summer; in these cases suitable *Citrus* seedlings for rootstock micrografting are necessary. All *Citrus* species are graft-compatible with each other and hence it may be possible to use them as rootstock for micrografting; however, no detailed comparative studies have been done (Chand et al., 2013). In addition, the potential for adventitious shoot formation and growth in decapitated seedlings of *Citrus* and *Poncirus* accessions is not known in various stages of seedling growth.

In *in vitro* culture, the potential for adventitious shoot formation in the epicotyl sections was different for different *Citrus* cultivars (Burger and Hackett, 1986) and the positions in epicotyls (Costa et al., 2004; Hussain et al., 2014). These results suggested that the potential for adventitious shoot formation in epicotyl explants was affected by the age of the seedlings, seedling genotype, composition of the culture medium and incubation conditions in *Citrus* (Costa et al., 2004).

In this study, to gather basic knowledge on the characteristics of seedlings from various *Citrus* accessions, adventitious shoot formation from the cut end of epicotyls and cotyledon axillary shoot formation were firstly investigated in one *Poncirus* and 11 *Citrus* accessions, including ‘Variegated Daidai’, that generates albino nucellar seedlings. If there is any relationship between the potential for shoot formation and micrografting success, it may be useful information when looking for accessions to generate rootstock seedlings for micrografting. Second, seedlings of various potential and different ages were used as rootstocks to examine the success rate in *in vivo* micrografting of satsuma mandarin. As a result, the seedlings of pummelo and pummelo hybrid accessions showed higher micrografting success rates and growth rates for satsuma mandarin compared with those of the other *Citrus* and trifoliate orange accessions.

Materials and Methods

Plant materials

Eleven open-pollinated *Citrus* accessions and one *Poncirus* accession (Table 1) were used to produce seedlings in this study. The 11 *Citrus* accessions were ‘Kashi papeda’ (*C. latipes* Tanaka), Bilolo (*C. montana* Tanaka), ‘Rangpur lime’ (*C. limonia* Osbeck), ‘Hirado-buntan’ pummelo (*C. maxima* (Burm.) Merr.), Natsudaiddai (*C. natsudaiddai* Hayata), ‘Zadaiddai’, ‘Kabusu’, and ‘Variegated Daidai’ sour oranges (*C. aurantium* Linn.), ‘Nansho-daidai’ (*C. taiwanica* Tanaka et Shimada), Yuzu (*C. junos* Sieb. ex Tanaka), Shiikuwasha (*C. depressa* Hayata), and the *Poncirus* accession was ‘Flying Dragon’ trifoliate orange (*P. trifoliata* (L.) Raf. var. *monstrosa*). These were grown in the orchard of Kyushu University Farm located in Sasaguri, Fukuoka Prefecture, Japan.

Table 1. Characteristics of the seeds of eleven *Citrus* accessions and one *Poncirus* accession used in this study.

No.	Accession	Scientific name (Tanaka's classification No.) ^z	No. of embryos per seed ^y (mean ± SD)	Weight of 10 seeds (g)
1	Kashi papeda	<i>C. latipes</i> Tanaka (10)	1.0 ± 0	4.48
2	Bilolo	<i>C. montana</i> Tanaka (29)	4.6 ± 0.5	1.90
3	Rangpur lime	<i>C. limonia</i> Osbeck (37)	2.0 ± 0.6	1.06
4	Hirado-buntan	<i>C. maxima</i> (Burm.) Merr. (56)	1.0 ± 0	3.66
5	Natsudaikai	<i>C. natsudaikai</i> Hayata (78)	4.0 ± 1.6*	2.36
6	Zadaikai	<i>C. aurantium</i> Linn. (93)	12.3 ± 4.1*	2.77
7	Kabusu	<i>C. aurantium</i> Linn. (93)	11.2 ± 5.6*	2.92
8	Variegated Daidai	<i>C. aurantium</i> Linn. (93)	4.2 ± 0.6	2.66
9	Nansho-daidai	<i>C. taiwanica</i> Tanaka et Shimada (87)	3.9 ± 2.0*	1.78
10	Yuzu	<i>C. junos</i> Siebold ex Tanaka (113)	4.9 ± 3.2*	3.74
11	Shiikuwasha	<i>C. depressa</i> Hayata (153)	16.7 ± 6.6*	2.10
12	Flying Dragon	<i>P. trifoliata</i> (L.) Raf. var. <i>monstrosa</i>	4.0 ± 2.5	2.78

^z Tanaka (1969).^y Value with asterisk (*) is cited from Ueno et al. (1967).

In each accession, including monoembryonic ‘Kashi papeda’ and ‘Hirado-buntan’, seedlings with the same morphological characteristics in terms of leaves, winged leaves, stems, stem internodes, and thorns were used and decapitated. Therefore, almost all seedlings used in the present study were nucellar, except for the zygotic seedlings of monoembryonic ‘Kashi papeda’ and ‘Hirado-buntan’ pummelo. Since self-incompatible ‘Hirado-buntan’ trees were cultivated in a pummelo germplasm orchard consisting of 50 pummelo accessions, almost all the seedlings used in the present study were considered intraspecific pummelo hybrids. The seeds of ‘Variegated Daidai’ generated a great number of albino seedlings and a small number of normal seedlings with green leaves considered to be zygotic. Only 2-week-old albino seedlings were used in this study, since their growth deteriorated from four weeks after germination and they later died.

Preparation of seedlings for adventitious shoot formation

Perfect *Citrus* accession seeds were collected from early November to late December from open-pollinated fruits grown in the orchard. Perfect Natsudaikai and ‘Nansho-daidai’ seeds were also collected in early June from open-pollinated fruits grown in the orchard. Large mature embryos were obtained from the perfect seeds of each *Citrus* accession, placed on wet filter paper and incubated in a growth chamber at 25 ± 2°C for one to two weeks. Vigorously germinating embryos with a root of 1–2 cm in length were transplanted to plastic trays (35 × 50 × 10 cm) containing a mixture of four soil types (Kanuma pumice:Bora pumice:Akadama soil (red clay ball):leaf mold = 1:1:1:1 v/v), and moved to a greenhouse to raise the seedlings. One hundred seedlings were planted per tray, and a total of about 540 seedlings were prepared for each accession. Two-week-

old, 4-week-old, 8-week-old, 4-month-old, and 8-month-old seedlings after germination (transplanting) were used.

‘Flying Dragon’ seeds were collected from mature fruit harvested in October, directly sown in wet sand and stratified under natural winter conditions to break down dormancy to obtain germinating seeds in spring the next year. The germinating seeds were transplanted to the seedling trays as described above. Two-week-old, 4-week-old, 8-week-old, and 4-month-old seedlings after germination (transplanting) were used.

Preparation of *Citrus* seedlings for micrografting

Perfect *Citrus* accessions seeds were collected in early April from the fruits of open-pollinated trees grown in the orchard, and the seedlings were produced according to the procedure mentioned above. The seedlings of ‘Flying Dragon’ that germinated in April were used. Two-week-old seedlings produced from seeds of open-pollinated fruits of five accessions (‘Hirado-buntan’, Natsudaikai, ‘Variegated Daidai’, Shiikuwasha, and ‘Flying Dragon’) were used for *in vivo* micrografting of ‘Okitsu-wase’ satsuma mandarin (*C. unshiu* Marrow.). Six seedlings were planted per pot with a volume of 600 mL.

Decapitation of seedlings for adventitious shoot formation

Vigorous seedlings were chosen from each group of 2-week-old, 4-week-old, 8-week-old, 4-month-old, and 8-month-old seedlings. Twenty-five to 30 seedlings at each age were decapitated at the lower, middle or upper positions of the epicotyls, respectively, with a sharp razor blade (Fig. 1). Seedlings with adventitious shoots appearing on the cut end of the epicotyls were observed 64 days after decapitation and shown as the percentage of seedlings with adventitious shoots. The days to ad-

Fig. 1. A 'Kabusu' seedling showing the upper, middle and lower epicotyl positions at which it was decapitated.

ventitious shoot initiation (more than 1 mm in length) and those to cotyledon axillary shoot initiation (more than 1 mm in length) were recorded every two days. The number of adventitious shoots per seedling was examined up to 64 days after decapitation. All of the cotyledon axillary shoots were removed after recording the above data to allow the growth of adventitious shoots on the cut end of epicotyls. The potential for adventitious shoot and cotyledon axillary shoot formation after decapitation of seedlings in *Citrus* and *Poncirus* accessions were evaluated as an index for shoot growth potential $[(A \times C + B \times D)/100]$ for each seedling age; here, "A" is the % of seedlings forming adventitious shoots, "C" is the number of adventitious shoots per seedling, "B" is the % of seedlings forming cotyledon axillary shoots and "D" is the number of cotyledon axillary shoots per seedling.

Minimum and maximum air temperatures in the greenhouse were 10°C in winter and 38°C in summer, respectively, during this 3-year experiment (2016–2019).

In vivo micrografting of satsuma mandarin

Thirty to 50 2-week-old, 4-week-old, 8-week-old, 4-month-old, and 8-month-old seedlings were chosen from each of eight accessions (Bilolo, 'Hirado-buntan', Natsudaikai, 'Kabusu', 'Zadaikai', 'Variegated Daidai', Shiikuwasha, 'Flying Dragon'), and were used as rootstocks for micrografting. In May, under a stereoscopic microscope, the satsuma mandarin shoot apex with six leaf primordia (about 0.6 mm in length) was excised from an axillary bud of a vigorously growing spring shoot with a length of about 5 cm. Three or four well-developed axillary buds on the upper half of the spring shoot were used for micrografting. Each of the seedlings was decapitated with a sharp razor blade leaving about 1 cm of the epicotyl from the cotyledon axillary

node. The shoot apex of each satsuma mandarin was placed with the cut end on the cambium seen in the cut end of epicotyl. A 1 cm epicotyl with the grafted shoot tip was wrapped up by expanded parafilm (Takahara et al., 1986). The graft union was carried to a growth chamber maintained at 25°C for one month and then transferred to a greenhouse. The air temperature in the greenhouse ranged from about 10°C to 38°C during this experiment. The successful rates were calculated 30 days after micrografting.

Histological observation

Histological observation of adventitious bud differentiation in the cut end of the epicotyl of 2-week-old decapitated seedlings was performed to determine its origin in stem tissues and the time course of tissue development. The leaving epicotyls of three types, i.e., lower, middle, and upper, were collected at 0, 4, 8, 16, 32, and 64 days after decapitation and fixed in FAA solution (70% ethanol:38% formaldehyde:glacial acetic acid, 90:5:5, v/v/v) for at least 12 hours. The fixed samples were dehydrated through a series of ethanol–butanol, and embedded in paraffin. Serial longitudinal sections of 8–13 µm thickness were made, stained in a solution of 0.25% Heidenhein's Hematoxylin, and observed and photographed under an optical microscope (E800; Nikon Corp., Tokyo, Japan).

Results

Adventitious shoot formation in decapitated seedlings

Multiple adventitious bud primordia appeared on the cambia of epicotyl cut ends in most decapitated seedlings of 11 accessions, except Bilolo, in which no adventitious bud was observed. It was difficult to count the exact number of adventitious buds initiated per decapitated seedling because they were very small, underdeveloped and had irregular morphology. Therefore, the number of adventitious shoots developing beyond 1 mm in length was examined in each decapitated seedling.

In 12 accessions, the average rates of decapitated seedlings producing adventitious shoots on the cut ends of epicotyls ranged from 0% for Bilolo to 100% for Natsudaikai (Table 2). In seedlings decapitated two weeks after germination (2-week-old seedlings), the highest average rate of decapitated seedlings producing adventitious shoots on the cut ends of epicotyls was found in Natsudaikai (100%), followed by Shiikuwasha (93.9%), 'Flying Dragon' (67.4%), 'Variegated Daidai' (57.8%), and 'Nansho-daidai' (52.2%). The highest rates in 8-week-old and 8-month-old seedlings were found in 'Flying Dragon' (55.3%) and 'Rangpur lime' (83.3%) respectively, and the rate in 1-year-old 'Flying Dragon' seedlings was 84.4% (unpublished data). The average rate of seedlings generating adventitious shoots was low in Yuzu and 'Zadaikai', ranging from 0% to 20.2% and from 0% to 4.9%, respectively.

In nine of the 12 accessions except Bilolo, 'Zadaikai'

Table 2. Adventitious shoot and cotyledon axillary shoot formation 64 days after decapitation in seedlings decapitated at various ages.

Accession (Period of germination)	Decapitated position in epicotyl	% of seedlings forming adventitious shoots when decapitated at indicated ages of seedlings ^z (A)					% of seedlings forming cotyledon axillary shoots when decapitated at indicated ages of seedlings ^y (B)				
		2-week- old	4-week- old	8-week- old	4-month- old	8-month- old	2-week- old	4-week- old	8-week- old	4-month- old	8-month- old
Kashi papeda (Late Oct.)	Lower	35.0	5.0	7.3	20.0	52.6	95.0	90.0	96.7	100	100
	Middle	45.0	0.0	13.3	4.0	81.0	87.0	92.0	80.0	96.0	58.0
	Upper	8.0	4.0	10.7	9.0	55.0	83.0	69.0	72.7	79.0	60.0
	Average	29.3	3.0	10.4	11.0	62.9	88.3	85.7	83.1	91.7	72.7
Bilolo (Middle Dec.)	Lower	0	0	0	0	—	100	100	84.0	100	—
	Middle	0	0	0	0	—	88.3	87.0	86.0	96.0	—
	Upper	0	0	0	0	—	95.0	88.0	88.3	92.0	—
	Average	0	0	0	0	—	94.4	91.7	86.1	96.0	—
Rangpur lime (Late Dec.)	Lower	32.0	8.0	9.0	0	90.0	96.0	96.0	87.0	93.3	80.0
	Middle	0	0	0	0	83.3	95.7	77.3	92.0	100	55.0
	Upper	28.0	0	3.3	28.0	76.6	96.0	92.0	76.7	92.7	73.3
	Average	20.0	2.7	4.1	9.3	83.3	95.9	88.4	85.2	95.3	69.4
Hirado-buntan (Middle Dec.)	Lower	36.0	0	4.0	0	39.3	96.0	100	100	85.0	88.0
	Middle	52.0	4.0	0	10.0	79.3	84.0	88.0	96.0	89.7	61.3
	Upper	38.5	12.0	0	5.0	74.7	47.0	20.0	92.6	66.7	30.0
	Average	42.2	5.3	1.3	5.0	64.4	75.1	69.3	96.2	80.7	72.9
Natsudaikai (Early Nov.)	Lower	100 ^x	0	0	5.0	13.3	100 ^x	100	92.0	100	93.3
	Middle	100 ^x	27.0	14.0	5.0	85.0	39.0 ^x	70.0	61.3	100	28.3
	Upper	100 ^x	68.0	21.3	8.0	90.0	25.3 ^x	23.0	17.3	82.0	36.7
	Average	100^x	31.7	11.8	6.0	62.8	54.7^x	64.3	56.9	94.0	52.7
Kabusu (Late Oct.)	Lower	8.0	0	0	5.0	6.6	96.0	74.7	73.0	95.0	86.7
	Middle	38.0	0	0	0	60.0	80.0	34.7	48.3	93.3	80.0
	Upper	30.7	0	20.0	0	50.0	54.0	11.3	30.0	71.6	40.0
	Average	25.6	0	6.6	1.7	38.9	76.7	40.2	50.4	86.7	68.9
Zadaikai (Early Jan.)	Lower	0	4.0	3.3	—	—	92.0	92.0	92.7	—	—
	Middle	0	0	0	—	—	81.0	90.3	96.0	—	—
	Upper	0	0	11.3	—	—	41.0	64.7	88.3	—	—
	Average	0	1.3	4.9	—	—	71.3	82.3	92.4	—	—
Variegated Daidai (albino nucellar) (Late Jan.)	Lower	16.3	—	—	—	—	83.7	—	—	—	—
	Middle	72.0	—	—	—	—	92.0	—	—	—	—
	Upper	85.0	—	—	—	—	50.0	—	—	—	—
	Average	57.8	—	—	—	—	73.9	—	—	—	—
Nansho-daikai (Late Dec.)	Lower	20.0 ^x	5.0	0	—	—	90.0 ^x	95.0	93.3	—	—
	Middle	63.3 ^x	5.0	0	—	—	76.7 ^x	65.0	95.0	—	—
	Upper	73.3 ^x	0	0	—	—	70.0 ^x	62.0	83.0	—	—
	Average	52.2^x	3.3	0	—	—	78.9^x	74.0	90.4	—	—
Yuzu (Early Nov.)	Lower	0	0	0	21.7	—	100	46.7	70.0	81.7	—
	Middle	0	0	0	20.0	—	100	76.7	80.0	100	—
	Upper	0	0	0	20.0	—	84.0	100	56.7	80.0	—
	Average	0	0	0	20.2	—	94.6	74.4	68.8	87.2	—
Shiikuwasha (Early Nov.)	Lower	95.0 ^x	0	0	0	0	100 ^x	88.7	76.0	80.0	87.7
	Middle	100 ^x	0	3.3	0	0	100 ^x	79.3	70.7	100	46.7
	Upper	86.7 ^x	0	0	0	0	95.0 ^x	72.0	76.3	79.0	48.1
	Average	93.9^x	0	1.1	0	0	98.3^x	80.0	74.3	86.3	60.8
Flying Dragon (Late Apr.)	Lower	50.1	24.0	25.0	40.0	—	84.0	92.0	87.0	95.0	—
	Middle	64.0	32.0	61.0	60.0	—	64.0	92.0	72.0	45.0	—
	Upper	88.0	24.0	80.0	20.0	—	72.0	80.0	82.0	60.0	—
	Average	67.4	26.7	55.3	40.0	—	73.3	88.0	80.3	66.7	—

^z In each decapitation treatment, 25–30 seedlings were used.^y In each decapitation treatment, 25–30 seedlings were used.^x These seedlings were germinated in early June.

—: No data.

and Yuzu, 2-week-old seedlings showed high rates of adventitious shoot formation (Table 2). The rates decreased with increases in the age of 8-week-old and 4-month-old seedlings, and increased in 8-month-old seedlings. 'Flying Dragon' showed relatively high rates of adventitious shoot formation at all different seedling ages.

In the 2-week-old seedlings of 'Kabusu', 'Variegated Daidai', 'Nansho-daidai', and 'Flying Dragon', the upper and/or middle positions of the epicotyls showed higher rates of adventitious shoot formation than the lower position, while in those of 'Kashi papeda', the middle and lower positions of the epicotyls showed higher rates than the upper position (Table 2). Although the three positions of epicotyls in 2-week-old seedlings of 'Rangpur lime', 'Hirado-buntan', Natsudaiddai, and Shiikuwasha showed similar rates for adventitious shoot formation, those in 4-week-old and 8-week-old seedlings of Natsudaiddai increased with increases in epicotyl length.

In all decapitated seedlings, the average number of days from decapitation to initiation of adventitious shoot growth ranged from 16.7 days for 2-week-old Natsudaiddai seedlings decapitated in early summer, to 64 days for 4-week-old 'Zadaiddai' seedlings decapitated in winter (unpublished data). Two-week-old Natsudaiddai, 'Nansho-daidai', and Shiikuwasha seedlings decapitated in early summer, and those of 'Variegated Daidai' decapitated in winter required relatively few days to initiation of adventitious shoot growth, ranging from 16.7 to 28.9 days. In 'Flying Dragon', about 40 days to adventitious shoot initiation were needed for 2-week-old to 4-month-old seedlings and 52 days in 1-year-old seedlings.

The average number of adventitious shoots per seedling ranged from 1.5 for 2-week-old seedlings of 'Kabusu' to 2.9 for 8-month-old seedlings of Natsudaiddai, except for two accessions, 'Kashi papeda' and 'Hirado-buntan', which ranged from 2.9 to 3.8 and 2.1 to 4.3, respectively (Table 3). In 2-week-old seedlings, the average number of adventitious shoots per seedling with adventitious shoots ranged from 1.5 for 'Kabusu' to 4.3 for 'Hirado-buntan', while it ranged from 1.0 for 'Kashi papeda' to 2.2 for 'Hirado-buntan' in 4-week-old seedlings. In 8-month-old seedlings, the average number increased with a range from 2.1 for 'Hirado-buntan' to 2.9 for 'Kashi papeda' and Natsudaiddai. The decapitated seedlings of 'Flying Dragon' did not show any obvious difference among the five decapitation ages, ranging from 1.7 to 2.3 adventitious shoots per seedling.

The average number of adventitious shoots per decapitated seedling was high in seedlings decapitated at an upper position of the epicotyl, followed by the middle and lower positions for 'Kashi papeda', Natsudaiddai, 'Variegated Daidai', 'Nansho-daidai', Shiikuwasha, and 'Flying Dragon' in 2-week-old seed-

lings. No clear differences were found among the three decapitated positions in the seedlings of the other accessions or the decapitation ages of the seedlings (Table 3).

In seedlings decapitated two weeks after germination, a few cell layers of the epicotyl cut end shrank and dried to form thin cell layers covering the cut end 16 days after decapitation (Fig. 2), while normal structure was observed on the day of decapitation. Several cell layers under the dried thin cell layers were empty and unstained; their nuclei and cytoplasm were degenerated and disappeared by day 16. In the epidermis and cortex, one or two cell layers adjacent to the degenerated empty cell layers were darkly stained, while in the cambium and pith, tissues several cell layers adjacent to the empty cell layers were darkly stained (Fig. 2). In the darkly stained cells on the cambium, mitotic figures were abundant. Adventitious buds initiating from these active mitotic cells on the cambium epicotyl cut end were observed 32 days after decapitation. In seedlings decapitated four weeks after germination or later, however, meristematic cells and calluses did not form in pith tissue due to lignification that formed a woody tissue; meristematic activity was seen in the cortex and mainly in the cells on the cambium, from which adventitious buds initiated.

Cotyledon axillary shoot growth in decapitated seedlings

In the decapitated seedlings, one or two cotyledon axillary shoots usually appeared before adventitious shoots appeared on the cut end of epicotyls. In the 12 accessions, the average rates of decapitated seedlings producing one or two cotyledon axillary shoots were relatively high with a range from 40.2% for 4-week-old seedlings of 'Kabusu' to 96.2% for 8-week-old seedlings of 'Hirado-buntan' (Table 2). The highest average rates in the accessions except for 'Variegated Daidai', were more than 86.7% for 4-month-old seedlings of 'Kabusu'. In 'Variegated Daidai', the average rate was 73.9% for 2-week-old albino seedlings. There was no relation between the highest average rate and decapitation age of seedlings in the 11 accessions. Generally, seedlings decapitated at a lower position on the epicotyls produced cotyledon axillary shoots with the highest rate, followed by those decapitated at the middle and upper positions; however, obvious differences were rarely detected in the rates among the three decapitation positions.

Micrografting of satsuma mandarin on seedlings with different potential for adventitious shoot growth

In eight accessions whose seedlings showed different potential for adventitious shoot formation after decapitation, the success rate of *in vivo* micrografting of satsuma mandarin was the highest in the seedlings of 'Variegated Daidai' (43.7%), followed by those of Natsudaiddai (33.3%), and 'Hirado-buntan' (22.2%),

Table 3. Number of adventitious shoots and cotyledon axillary shoots per seedling when decapitated at various seedling ages 64 days after decapitation.

Accession (Period of germination)	Decapitated position on epicotyl	Mean No. of adventitious shoots forming in decapitated seedlings at the indicated seedling age ^z (C)					Mean No. of cotyledon axillary shoots forming in seedlings decapitated at the indicated seedling age ^y (D)				
		2-week- old	4-week- old	8-week- old	4-month- old	8-month- old	2-week- old	4-week- old	8-week- old	4-month- old	8-month- old
Kashi papeda (Late Oct.)	Lower	2.1	1.0	5.5	4.0	1.9	1.8	1.5	1.8	1.9	1.8
	Middle	2.6	0	2.3	6.0	3.1	1.3	2.0	1.8	1.8	1.7
	Upper	4.5	1.0	2.7	1.5	3.6	1.3	1.5	1.8	1.7	1.4
	Average	3.1	1.0	3.5	3.8	2.9	1.5	1.7	1.8	1.8	1.6
Bilolo (Middle Dec.)	Lower	0	0	0	0	—	1.9	1.7	1.6	1.8	—
	Middle	0	0	0	0	—	1.8	1.6	1.6	1.6	—
	Upper	0	0	0	0	—	1.8	1.6	1.6	2.0	—
	Average	0	0	0	0	—	1.8	1.6	1.6	1.8	—
Rangpur lime (Late Dec.)	Lower	1.7	1.5	3.5	0	1.6	1.8	1.8	1.9	1.7	1.9
	Middle	0	0	0	0	3.1	1.9	1.7	1.9	1.7	1.6
	Upper	1.4	0	3.0	1.0	2.8	1.9	1.7	1.9	2.0	1.5
	Average	1.6	1.5	2.2	1.0	2.5	1.9	1.8	1.9	1.8	1.6
Hirado-buntan (Middle Dec.)	Lower	5.1	0	3.0	0	1.7	1.9	1.8	1.8	1.9	1.8
	Middle	5.1	1.0	0	2.3	2.2	1.7	1.7	1.7	2.0	1.6
	Upper	2.7	3.3	0	3.0	2.3	1.5	1.2	1.6	1.9	1.6
	Average	4.3	2.2	3.0	2.7	2.1	1.7	1.6	1.7	1.9	1.7
Natsudaikai (Early Nov.)	Lower	1.4 ^x	0	0	2.0	1.5	1.9 ^x	1.6	1.7	2.0	1.9
	Middle	1.8 ^x	1.7	1.0	1.0	3.5	1.1 ^x	1.6	1.6	1.9	1.2
	Upper	2.7 ^x	1.8	1.2	1.5	3.6	1.3 ^x	1.2	1.6	1.8	1.6
	Average	2.0^x	1.8	1.1	1.5	2.9	1.4^x	1.5	1.6	1.9	1.5
Kabusu (Late Oct.)	Lower	1.0	0	0	1.0	2.0	1.8	1.5	1.7	1.7	1.4
	Middle	1.9	0	0	0	2.7	1.4	1.1	1.9	1.6	1.7
	Upper	1.5	0	1.0	0	2.0	1.4	1.0	1.7	1.6	1.5
	Average	1.5	0	1.0	1.0	2.2	1.6	1.2	1.8	1.6	1.5
Zadaikai (Early Jan.)	Lower	0	1.0	1.0	—	—	1.8	1.7	1.9	—	—
	Middle	0	0	0	—	—	1.6	1.7	1.7	—	—
	Upper	0	0	1.7	—	—	1.8	1.5	1.5	—	—
	Average	0	1.0	1.7	—	—	1.7	1.6	1.7	—	—
Variegated Daidai (albino nucellar) (Late Jan.)	Lower	1.3	—	—	—	—	1.4	—	—	—	—
	Middle	1.6	—	—	—	—	1.7	—	—	—	—
	Upper	2.4	—	—	—	—	1.3	—	—	—	—
	Average	1.8	—	—	—	—	1.5	—	—	—	—
Nansho-daidai (Late Dec.)	Lower	2.0 ^x	2.0	0	—	—	1.8 ^x	1.7	1.3	—	—
	Middle	2.4 ^x	1.0	0	—	—	1.6 ^x	1.6	1.6	—	—
	Upper	2.5 ^x	0	0	—	—	1.6 ^x	1.6	1.6	—	—
	Average	2.3^x	1.5	0	—	—	1.7^x	1.7	1.5	—	—
Yuzu (Early Nov.)	Lower	0	0	0	2.3	—	1.6	1.7	1.7	1.8	—
	Middle	0	0	0	1.7	—	1.4	1.7	1.6	1.8	—
	Upper	0	0	0	3.0	—	1.5	1.6	1.6	1.6	—
	Average	0	0	0	2.3	—	1.5	1.7	1.6	1.8	—
Shiikuwash (Early Nov.)	Lower	1.6 ^x	0	0	0	0	1.8 ^x	1.8	1.5	1.4	1.8
	Middle	2.1 ^x	0	1.0	0	0	1.7 ^x	1.8	1.4	1.6	1.8
	Upper	2.5 ^x	0	0	0	0	1.6 ^x	1.7	1.5	1.5	1.6
	Average	2.1^x	0	1.0	0	0	1.7^x	1.8	1.5	1.5	1.8
Flying Dragon (Late Apr.)	Lower	1.9	1.7	1.3	1.8	—	1.9	1.8	1.5	1.8	—
	Middle	2.3	1.6	2.1	1.8	—	1.7	1.7	1.7	1.6	—
	Upper	2.6	2.0	1.9	1.5	—	1.8	1.6	1.4	1.3	—
	Average	2.3	1.8	1.8	1.7	—	1.8	1.7	1.5	1.6	—

^z Adventitious shoots with a height over 1.0 mm were counted.^y Cotyledon axillary shoots with a height over 1.0 mm were counted.^x These seedlings were germinated in early June.

—: No data.

whereas it was low in Shiikuwasha (9.4%) and ‘Flying Dragon’ seedlings (8.0%) (Table 5). The rates de-

Fig. 2. Sections of the epicotyls of ‘Kabusu’ sour orange seedlings decapitated two weeks after germination (A) 0 day after decapitation, (B) cut end of the epicotyl 0 days after decapitation, (C) showing high mitotic activity in the meristematic cells of the cambium close to the cut end of the epicotyl 16 days after decapitation, (D) magnification of meristematic cells observed in (C), (E) adventitious bud formation on the cut end of the epicotyl 32 days after decapitation, and (F) *in vivo* adventitious shoots in a decapitated epicotyl 64 days after decapitation. **ab**, adventitious bud; **ca**, cambium; **co**, cortex; **dc**, degenerated cells; **e**, epidermis; **mc**, meristem cells on cambium; **o**, oil gland; **p**, pith; **dsc**, degenerated and shrunken cells; **wt**, woody tissue. Black Bars = 50 μ m and white Bars = 1 mm.

creased with increasing seedling age. All scions grafted on 4-month-old ‘Kabusu’ and ‘Flying Dragon’ seedlings died by day 30, while 13.3% of shoot tips grafted on 4-month-old ‘Hirado-buntan’ survived and grew; however, micrografting with 8-week-old rootstock seedlings was not successful, probably because of technical difficulty due to the development of woody tissues. When the scions micrografted on the rootstock seedlings turned brown or died, adventitious shoots and/or axillary shoots formed. When the decapitated albino seedlings of ‘Variegated Daidai’ were used as rootstocks, micrografting success was confirmed by the green color of growing satsuma mandarin shoots, while failure was determined by the white color of adventitious shoots originating from the decapitated albino seedlings of ‘Variegated Daidai’ (Fig. 3). When the scion developed into a shoot, adventitious buds (shoots) were not observed after micrografting. In five accessions, the number of days to initiation of shoot growth (more than 1 mm in length) was the lowest (7.7 days) in satsuma mandarin scions micrografted on Natsudaiddai seedlings and the longest (16.3 days) in those grafted on ‘Hirado-buntan’ seedlings. Growth of micrografted satsuma mandarin at day 50 was the fastest for Natsudaiddai seedlings followed by ‘Variegated Daidai’ albino seedlings, while it was slow for ‘Flying Dragon’ seedlings (unpublished data). In summary, the seedlings of ‘Hirado-buntan’ pummelo and Natsudaiddai and ‘Variegated Daidai’ pummelo relatives showed higher success rates and growth rates for micrografting of satsuma mandarin in comparison to those of the other *Citrus* and trifoliate orange accessions.

Discussion

Although the average rates of seedlings generating adventitious shoots tended to decrease with increases in

Table 4. The potential for adventitious shoot and cotyledon axillary shoot formation after decapitation of seedlings in eleven *Citrus* accessions and one *Poncirus* accession.

Accession	Index for shoot growth potential $[(A \times C + B \times D)/100]^z$ at the indicated decapitation age of seedlings				
	2-week-old	4-week-old	8-week-old	4-month-old	8-month-old
Kashi papeda	2.23	1.45	1.80	2.07	2.99
Bilolo	1.74	1.47	1.38	1.73	—
Rangpur lime	2.14	1.63	1.71	1.87	3.19
Hirado-buntan	3.10	1.23	1.67	1.67	2.85
Natsudaiddai	2.77	1.20	1.06	1.80	2.61
Kabusu	1.61	0.48	0.92	1.40	1.89
Zadaiddai	1.21	1.33	1.65	—	—
Variegated Daidai	2.14	—	—	—	—
Nansho-daidai	2.54	1.31	1.36	—	—
Yuzu	1.42	1.26	1.10	1.83	—
Shiikuwasha	3.64	1.44	1.13	1.29	0.85
Flying Dragon	2.87	1.98	2.20	1.75	—

^z A and B: average value listed in Table 2; C and D: average value listed in Table 3.

—: No data.

Table 5. The success rates of *in vivo* micrografting of satsuma mandarin shoot tips with 6-leaf primordia on different rootstock seedlings 30 days after micrografting and maintained at 25°C.

Accession	Success rate of micrografting of shoot tips with 6-leaf primordia at the indicated age of seedlings used for micrografting (%) ^z				
	2-week-old	4-week-old	8-week-old	4-month-old	8-month-old
Bilolo	—	13.0	3.8	—	—
Hirado-buntan	22.2	9.7	13.3	13.3	0
Natsudaikai	33.3	20.1	4.3	—	—
Kabusu	3.8	0	3.3	0	—
Zadaikai	16.7	4.0	—	—	—
Variegated Daidai	56.2	—	—	—	—
Shiikuwasha	9.4	13.8	6.7	—	—
Flying Dragon	8.0	3.0	0	0	—

^z In each combination, 25–30 seedlings were used for *in vivo* micrografting.

—: No data.

Fig. 3. *In vivo* micrografting of satsuma mandarin on 2-week-old albino seedlings of ‘Variegated Daidai’ 30 days after micrografting. (A) A green shoot of satsuma mandarin showing successful micrografting; (B) an albino adventitious shoot of an albino seedling showing unsuccessful micrografting. Horizontal Bars = 5 mm.

the average rates of seedlings generating cotyledon axillary shoots in the 12 accessions, it is clear that in the decapitated seedlings of all accessions, cotyledon axillary shoots appeared relatively constantly with a high frequency of recovering seedling growth after decapitation. It appears that this phenomenon is universal to the decapitated seedlings of *Citrus* and *Poncirus* plants. On the other hand, the present result suggested that the rates of seedlings generating adventitious shoots in the cut end of epicotyls is genotype-specific, although the rates were different in three sour orange cultivars ‘Kabusu’, ‘Zadaikai’, and ‘Variegated Daidai’ (Table 2). Sour orange cultivars have been reported to be either genetically identical clones or sour orange hybrids (Siragusa et al., 2006).

In the present study, the rates of decapitated seedlings generating adventitious shoots on the cut edge increased with increases in the length of epicotyls after decapitation. A similar result was reported by Costa

et al. (2004), who showed that in *in vitro* culture of epicotyl sections from ‘Rangpur lime’ and grapefruit seedlings, the potential for adventitious shoot regeneration increased as the distance of the explants from the cotyledonary node increased. They also suggested that the potential was affected by the age of the seedlings, plant material and incubation conditions, as seen in the present study. Dejam et al. (2006) reported that the growth substance BA induced adventitious bud initiation in *Citrus* epicotyl explants and that addition of low levels of auxin to the media enhanced adventitious shoot growth. It has also been reported that BA added to the medium increased the number of buds and subsequent shoot formation in epicotyl explants in *Citrus* (Garcia-Luis et al., 1999). Park and Son (1988) reported that the transfer of endogenous hormones to the site of injury in *Populus nigra* leaves *in vitro* resulted in a suitable level of growth promoters for regeneration. A similar mechanism reported in these studies may regulate adventitious shoot formation in decapitated epicotyls *in vivo*.

The gradient for adventitious shoot formation seen in the cut edge of epicotyls in *Citrus* and *Poncirus* accessions is an excellent option as a plant growth strategy for rapid recovery of seedling growth when the shoots above the epicotyls have been lost by accident. The role of the epicotyl is to raise the leaves up in the air so that they are exposed to sufficient sunlight. Thus, it seems that the longer the remaining epicotyls, the more advantageous it is to form adventitious shoots, while the shorter the leaving epicotyls, the more advantageous it is to form cotyledon axillary shoots.

It is considered that the high rates for adventitious shoot formation in 2-week-old seedlings are due to the high potential of cotyledons to supply their storage nutrients for the initiation of adventitious buds and subsequent shoot growth. In the seedlings older than two weeks, the size and storage nutrients in their cotyledons decreased with growth and resulted in lower rates of

adventitious shoot formation. Tezuka et al. (2011) reported that young *in vivo* tomato seedlings decapitated at the epicotyl more actively regenerated adventitious shoots on the cut end of epicotyls than older decapitated ones as observed in *Citrus* seedlings. The 8-month-old seedlings with more than ten leaves showed high rates of adventitious shoot formation. This result is probably due to photosynthetic products that are accumulated in their epicotyls and roots from which a sufficient amount of nutrients is supplied for the initiation of adventitious buds and subsequent shoot growth.

In the greenhouse, short days and relatively low temperature during the winter in 2-week-old to 4-month-old seedlings with poor root systems, and long days and optimal temperature in spring and summer in 8-month-old seedlings with developed root systems also affected the photosynthetic products and the amount of storage nutrients in their roots, eventually affecting the initiation rate of adventitious shoots. It is also suggested that 'Flying Dragon' seedlings decapitated under the appropriate environmental conditions during spring and summer resulted in relatively high rates for adventitious shoot initiation through different seedling ages.

Because the days to adventitious shoot initiation were influenced by the temperature change in the four seasons, this factor is not used to estimate the potential for adventitious shoot formation in decapitated seedlings. Based on the results of 2-week-old seedlings in the 12 accessions, the extent of the potential for adventitious shoot formation in decapitated seedlings is estimated as follows in descending order: Natsudaidai (2.00), Shiikuwasha (1.97), 'Hirado-buntan' (1.82), 'Flying Dragon' (1.55), 'Nansho-daidai' (1.20), 'Variegated Daidai' (1.37), 'Kashi papeda' (0.91), 'Kabusu' (0.36), 'Rangpur lime' (0.32), Yuzu (0.0), 'Zadaidai' (0.0), and Bilolo (0.0). Here, the index in parentheses is calculated following the formula: average % seedlings forming adventitious shoots (A in Table 2) $\times$ average No. of adventitious shoots (C in Table 3). This index represents the number of adventitious shoots that can be generated from one seedling decapitated two weeks after germination. None of the decapitated seedlings of Bilolo, a hybrid between 'Kashi papeda' and mandarin, formed adventitious shoots after decapitation, suggesting that it does not have potential for adventitious shoot formation. The potential is thought to be genotype-specific as indicated by Costa et al. (2004) in citrus and Tezuka et al. (2011) in tomato.

The indices for the potential to support both adventitious shoot growth and cotyledon axially shoot growth after decapitation were also calculated for each seedling age and listed in Table 4. This index reveals the number of shoots that can be generated from one decapitated seedling, or the potential of seedlings to support initial shoot growth. In the 2-week-old seedlings of 12 accessions, those that showed high potential were

Shiikuwasha (3.64), 'Hirado-buntan' (3.10), 'Flying Dragon' (2.87), Natsudaidai (2.77), 'Nansho-daidai' (2.54), 'Kashi papeda' (2.23), and 'Variegated Daidai' (2.14) in descending order. Of the eight accessions examined, 'Variegated Daidai', Natsudaidai and 'Hirado-buntan' seedlings showed high success rates for micrografting of satsuma mandarin, whereas 'Kabusu', 'Flying Dragon', and Shiikuwasha seedlings resulted in very low success rates. This result suggests that there is no relationship between the potential for adventitious shoot formation and micrografting success, and that shoot formation is not a useful marker when assessing accessions to generate rootstock seedlings for micrografting.

The rapid initiation of growth (7.7 days after grafting) of satsuma mandarin shoot tips grafted on Natsudaidai seedlings corresponds well with the result that the number of days to adventitious shoot initiation (16.7 days) was the lowest of all accessions examined (unpublished data). This result suggests that the effect of the rootstock on the scion growth rate may be inferred from the days to initiation of adventitious shoot formation. Although albino seedlings of 'Variegated Daidai' ceased growing by about four weeks after germination, 57.8% of albino seedlings decapitated two weeks after germination generated adventitious shoots on the cut end of epicotyls. Interestingly, 56.2% of the 2-week-old albino seedlings on which satsuma mandarin shoot tips were micrografted resulted in success. This suggests that 2-week-old albino seedlings have sufficient graft-compatibility with satsuma mandarin shoot tips and high potential to support scion growth after micrografting, as estimated from the index of potential for adventitious shoot and cotyledon axially shoot formation.

Hussain et al. (2014) and Chand et al. (2016) indicated that the success of *in vitro* micrografting depended on the genotype of rootstock seedlings. Despite their high index for adventitious shoot formation, however, Shiikuwasha and 'Flying Dragon' seedlings showed low success rates for satsuma mandarin micrografting (Table 5). It has been reported that *in vivo* micrografting of satsuma mandarin on trifoliate orange seedlings resulted in a success rate of 20–50% (Ohta et al., 2011; Takahara et al., 1986). Ohta et al. (2011) indicated that it is very difficult for untrained personnel to achieve high success rates using traditional methods because micro-techniques and occasional aseptic manipulation are required. Using 'Hirado-buntan', Natsudaidai or 'Variegated Daidai' seedlings for satsuma mandarin micrografting is easier for untrained personnel than using trifoliate orange seedlings. The present result suggests that instead of trifoliate orange seedlings, the seedlings of pummelo or pummelo hybrids like Natsudaidai and 'Variegated Daidai' will be useful for micrografting not only in temperate regions, but also in tropical regions. These seedlings may be

useful to achieve high success rates not only for satsuma mandarin, but also for various *Citrus* cultivars. On the other hand, Bilolo, Yuzu, and ‘Zadaidai’ seedlings showed low micrografting success rates, but low potential for adventitious shoot formation. This is also interesting as there is almost no need to distinguish scion shoots from adventitious shoots from rootstock seedlings.

Conclusion

Trifoliate orange with resistance to the tristeza virus and cold hardiness is an exclusive rootstock for satsuma mandarin in Japan (Kawase et al., 1987), but the mature seeds require cold temperature to germinate, and thus it is difficult to use them for micrografting in tropical and subtropical regions. In these regions, fresh and mature citrus seeds are available for micrografting onto rootstocks throughout the year. Thus, instead of trifoliate orange seedlings, using seedlings of pummelo or pummelo hybrids like Natsudaidai and ‘Variegated Daidai’ for micrografting is expected. These seedlings may be useful not only for high success rates with satsuma mandarin, but also for various *Citrus* cultivars. Once a virus-free shoot is established, it will be possible to mass propagate it on the appropriate rootstock.

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