Semi-sterilized Tissue Culture for Rapid Propagation of Grapevines (*Vitis vinifera* L.) Using Immature Cuttings

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Abstract. Rapid expansion of grapevine plantings in many parts of the world has led to increased demand for desirable planting stocks. In countries that rely on importing new varieties and have strict quarantine rules, such as Australia, vines need to stay under quarantine for ≈ 2 years before they are released, at which time there is very limited wood available. Hence, rapid expansion of propagating stock after release is the key to multiplying up new varieties. A novel method, referred to as Semi-sterilized Tissue Culture (SSTC) using immature single-node cuttings, was established and evaluated as a way of rapid expansion of grapevine (*Vitis vinifera* L.) planting stock. In the SSTC method, immature single-node cuttings were surface-sterilized using methylated spirits and then cultured in the root pulsing medium [1/2 Murashige and Skoog (MS) medium supplemented with 40 μM indole-3-butyric acid (IBA)] for 24 hours. They were then planted in sterilized aerobic rooting medium (sphagnum peat:coarse river sand:perlite = 0.5:1:2) and cultured in a tissue culture room for ≈ 4 weeks for root initiation and development. The rooted immature single-node cuttings were then transferred to normal propagation beds in a greenhouse and potted on for acclimatization. Tube stock generated by SSTC easily acclimatized with a 15 times higher root strike rate than cutting propagation. It also took at least 50% less time than fully sterilized micropropagation methods to produce planting stocks. The advantages of the SSTC method are that it can be conducted under semisterilized conditions, avoiding degeneration and bacterial contamination problems encountered in micropropagation methods. By removing the time-consuming steps of the explant establishment, proliferation, and maintenance in vitro, the propagation process was simplified compared with conventional sterile tissue culture procedures. The SSTC procedure removed the need for high operator skill levels, reducing expense and allowing easier commercial adoption.

Increasing international market demand for wine and grapes has resulted in expanded plantings in many parts of the world. One consequence in some countries has been a shortfall in desirable planting stocks. This is especially true where the industry is based on imported cultivars such as in New Zealand, South Africa, Chile, India, China, and Australia with strict quarantine regulations governing the importation of grapevines.

The Australian wine and grape industry has developed and greatly benefited from

selection of imported clonal material suited to particular Australian wine grape-growing areas (Dry, 2004). Having new clones available as quickly as possible and being able to multiply these new varieties up for further propagation to supply growers is critical to keeping Australia at the cutting edge of varietal development. However, Australia has strict quarantine rules, which require imported cuttings to be held under quarantine for ≈ 2 years until they pass virus indexing (Anonymous, 2013). Grapevine virus indexing is a process of visual disease screening when the imported dormant cuttings are rooted and grown in closed quarantine facilities at government post-entry quarantine establishments. On release from quarantine, very limited cane material is available for cuttings. Consequently, it takes several years to obtain sufficient material to build up numbers sufficient to establish new planting blocks using traditional propagation procedures.

Traditional propagation methods, involving the establishment of mother vines from which dormant cuttings are taken for rooting or bench grafting, are relatively slow and not suited to rapid multiplication for commercial cultivation for newly released cultivars (Singh et al., 2004). Mother vines are generally planted

from dormant rootlings or potted plants. Commercial-scale numbers of cuttings are not produced from mother vines for at least 3 years depending on the variety (Walker and Golino, 1999).

Mist propagation of herbaceous cuttings has been practiced for rapid propagation of grapevines (California Association of Winegrape Growers, 1998). This method involves collecting multiple green cuttings and rooting them under mist. Warmth, humidity, and light are critical for success with this technique. The cuttings work best with at least two nodes and two lateral buds. The cuttings' maturity is critical for rooting. Very young or greatly lignified cuttings often fail to root (Walker and Golino, 1999). Semimature partially lignified cuttings are required for this method. This method provides a possibility to use immature cuttings for propagation; however, the multiplication rate could be improved if single-node immature cuttings could be used and if both the semimature partially lignified and softer cuttings could be used.

In vitro micropropagation is an economically feasible method for rapid multiplication of desirable plant genotypes (Gray and Fisher, 1985; Murashige, 1974; Read, 2007) and has been suggested as a means of propagating scarce or hard-to-propagate planting stock of grape clones and varieties (Gray and Fisher, 1985; Lee and Wetzstein, 1990; Lewandowski, 1991; Singh et al., 2004; Walker and Golino, 1999). From the 1970s to early 1990s, success was reported in vitro multiplication in grapes using different explants such as shoot apices, axillary bud, meristem, etc. (Barlass and Skene, 1978; Gray and Benton, 1991; Harris and Stevenson, 1982; Novak and Jůvova, 1983), but the commercial aspect was not worked out in most cases with problems of vitrification of many small shoots and callus formation at the base of the cultures (Singh et al., 2004). Later, grape plantlet multiplication was achieved through axillary shoot proliferation using protocols developed in muscadine grapes (Thies and Graves, 1992; Torregrossa and Bouguet, 1995) and in *V. vinifera* grapes (Mhatre et al., 2000), but this technique was not reproducible when commercially tested (Singh et al., 2004). In the 2000s, a technique of rapid in vitro multiplication of *V. vinifera* L. cultivars was developed, which enabled fast multiplication of thousands of plantlets per annum (Singh et al., 2004). In our micropropagation of the imported grape variety ‘Pinot Gris’, to improve rooting in microcuttings harvested from in vitro culture, we modified the micropropagation protocol by changing the conventional agar rooting medium to an aerobic rooting medium, an autoclaved propagation mix composed of sphagnum, peat, coarse sand, and perlite (Newell et al., 2003, 2005). Using this technique, more than 2000 rooted vines of ‘Pinot Gris’ were produced within 2 years from limited material from mother vines (Shan and Seaton, 2007). Although the micropropagation technique is straightforward (Monette, 1988), except for an established infrastructure, it needs practiced skills requiring substantial

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amounts of time and labor (Walker and Golino, 1999). In addition, during our micropropagation of 'Pinot Gris' and other varieties such as 'Merlot 181', it was observed that in vitro explants would easily turn yellow and die after a couple of subcultures. This problem has also been reported on micropropagation of *V. vinifera* L. cv. Napoleón (Ibáñez et al., 2003). Other considerations of this method are control of plant-associated bacterial contamination and the labor required for frequent (often at 2-week intervals) subculturing.

Rapid propagation can significantly speed up supply of a large amount of planting stock and offer large economic advantages (Walker and Golino, 1999). Therefore, development of rapid propagation techniques may offer a solution to improvement in grapevine propagation where scarce stock plant material is available such as plants received through quarantine. The purpose of this study was to develop and evaluate a novel technique termed SSTC for fast propagation of planting stocks of imported grapevines using immature single-node cuttings from limited source material. Central to this technique was the elimination of the need for explants' initiation and in vitro maintenance as required by micropropagation tissue culture protocols and the need for multinodal cuttings by use of single fresh nodes as compared with ex vivo cuttings propagation techniques.

Materials and Method

Plant materials and preparation

All the plant propagation materials were maintained in pots in a greenhouse at the Department of Agriculture and Food, Western Australia, South Perth. The grapevines (*Vitis vinifera* L.) tested in experiments were sourced from plants that had passed through quarantine after importation by the wine industry. A total of 10 genotypes of wine grapes (*V. vinifera* L.) were tested: 'Verdejo' clone; 'Pinot Gris' clone; 'Pinot Noir' clone; 'Tempranillo' clone A; 'Tempranillo' clone B; 'Tempranillo' clone C; 'Tempranillo' clone D; 'Tempranillo' clone E; 'Merlot' clone; and a 'Savagnin' clone.

Immature single-node cutting preparation

Young shoots of 2 to 6 mm thickness in diameter were harvested from mother plants. Shoots were cut into 3 to 4 cm long single node cuttings and leaves removed. For the cuttings from the terminal shoot tip of a cane, one to two very small leaves remained intact.

Experiments

Experiments consisted of: 1) establishment of SSTC method; 2) comparison on rooting effect between cuttings propagation and SSTC method; and 3) application of SSTC protocol to different genotypes.

Expt. 1: Establishment of SSTC method. The genotype 'Verdejo' clone was used in this experiment. The SSTC method involved surface sterilization of immature single-node cuttings, root pulsing, root development, and acclimatization.

Surface sterilization. The immature single-node cuttings were immersed in 0.1% Tween 80 (Rowe Scientific Pty. Ltd.) and shaken for 2 min and rinsed under running tap water for 30 min. Then three different sterilization treatments of deionized (DI) water (control), 70% methylated spirits, and 70% methylated spirits followed by 0.5% chlorine treatment were tested for effectiveness (Table 1). The experiment was a completely randomized design with a minimum 10 cuttings per treatment replicated three times.

Root pulsing. After sterilization, the immature single-node cuttings were inserted base first in root pulsing medium to a depth of ≈ 15 mm and then kept in the dark at 20 °C for 24 h. The root pulsing medium was half-strength MS (Murashige and Skoog, 1962) with 10 g·L⁻¹ sucrose and 8 g·L⁻¹ grade J3 powdered agar (Gelita Australia Pty. Ltd.) with pH adjusted to 6.5 before autoclaving and supplemented with 40 μ M IBA.

Root development. After root pulsing, immature single-node cuttings were planted in sterilized punnets (7 × 14 × 5 cm) containing an aerobic rooting medium made by autoclaving a propagation mix composed of sphagnum peat, coarse river sand (1 to 3 mm diameter), and perlite (horticulture-grade, P500, large grain) in a ratio of 0.5:1:2 with a pre-autoclave pH of 6. Punnett were put in a clean non-sterilized food container (under sterile conditions) and placed in a culture room maintained at a constant temperature of 22 °C with a 16-h photoperiod at light intensity of 50 μ mol·m⁻²·s⁻¹ for ≈ 4 weeks until roots developed (Newell et al., 2003, 2005). After 4 weeks, the immature single-node cuttings were assessed for rooting rate, quality, death, and contamination rate.

Rooted vine acclimatization. Punnett with rooted immature single-node cuttings were placed in a nursery tray and transferred onto a sand propagation bed with

bottom heated to 24 to 26 °C. Clough covers (wet muslin cloth) were placed over the sand beds holding the plant punnets to maintain humidity while reducing excessive water being deposited on cuttings. In the propagation house, a misting system was programmed to come on every few minutes to maintain a damp film on the clough covers. Humidity was maintained at $\approx 80\%$ under the clough covers. The rooted cuttings remained there for 15 to 20 d, and 5 to 7 d before moving, the cuttings were potted up using a mix of polystyrene grit: perlite:peat = 1:1:0.5 to 1 in tree tubes (12 cm long). The tube potted vines were then moved off the propagation bed into the greenhouse under 50% living shade. The vines were considered ready for delivery after 17 to 25 d when they were 15 to 20 cm long with five to seven fully expanded leaves as shown in Figure 1.

Statistical analysis and best protocol determination. A generalized linear model was applied assuming binomial distribution of data (i.e., immature single-node cuttings rooted/total number of immature single-node cuttings tested) to compare the treatments (Payne, 2010). Analysis of variance was conducted and *t* tests were used to compare against the control using GenStat for Windows, Edition 16 (<<http://www.genstat.com>>). DI water was used as a control, to which other treatments were compared. The best protocol was determined in Expt. 1 and used in subsequent experiments as the established SSTC method.

Expt. 2: Comparison of rooting effect between cuttings propagation and SSTC method. A total of 40 immature single-node cuttings of 'Tempranillo' clone A were used for cuttings propagation. The experiment was laid out in a complete randomized design with 10 immature single-node cuttings per treatment replicated four times. Immature single-node cuttings were dipped in 3 g·L⁻¹ Clonex rooting hormone gel (Growth Technology, Western Australia) and planted in propagation potting mix (polystyrene grit: perlite:peat = 1:1:0.5 to 1) contained in plant punnets. The punnets were put into a nursery tray and placed on sand propagation beds with the bottom heated to 24 to 26 °C. The propagation beds were covered with a clough, which was kept damp to provide a high-humidity environment. These beds were in a mist house, which had a 75% of living shade. The established SSTC method was used as a control. A total of 40 immature single-node cuttings of 'Tempranillo' clone A were used with four replicates of 10

Table 1. Sterilization treatments of 'Verdejo' clone immature single-node cuttings.

	Number of replicates	Number of cuttings treated	Treatment description
DI water (control)	3	34	Rinsed with sterilized deionized (DI) water 4 to 5 times and then planted into root pulsing medium
Methylated spirits	3	33	Immersed in 70% methylated spirits (Diggers, Australia) and shaken for 30 s, rinsed with sterilized DI water 4 to 5 times, and then planted into root pulsing medium
Methylated spirits + chlorine	3	45	Immersed in 70% methylated spirits and shaken for 30 s followed with immersing in 0.5% liquid pool chlorine (Premier Chlor, Australia) and shaken for 20 min, during which vacuum once for 1 min, rinsed with sterilized DI water 4 to 5 times, and then planted into rooting pulsing medium

immature single-node cuttings per replicate. Rooting rate, quality, and rotting/death rate were recorded 3 weeks after treatments.

Statistical analysis. A generalized linear model was applied assuming binomial distribution of data (e.g., number of cuttings rooted/total number of cuttings tested) to compare the treatments (Payne, 2010). Analysis of variance was conducted and *t* tests were used to compare with the control using GenStat for Windows, Edition 16 (<http://www.genstat.com>). The SSTC method was used as a control, to which the cuttings propagation method was compared.

Expt. 3: Application of SSTC protocol to other genotypes. The SSTC method was applied to a total of 887 immature single-node cuttings for 10 genotypes. The experiment was laid out in a complete randomized design with approximately one-third of the total number of immature single-node cuttings per treatment replicated three times. Eighty-five immature single-node cuttings from the 'Verdejo' clone were used as a control. Other cuttings were 80 immature single-node cuttings from 'Pinot Gris'; 83 from 'Pinot Noir'; 96 from 'Tempranillo' clone A; 128 from 'Tempranillo' clone B; 96 from 'Tempranillo' clone C; 95 from 'Tempranillo' clone D; 70 from 'Tempranillo' clone E; 30 from 'Merlot' clone; and 124 from 'Savagnin' clone. Rooting rate, quality, death rate, and contamination rate were measured 4 weeks after treatments.

Statistical analysis. A generalized linear model was applied assuming binomial distribution of data (e.g., number of cuttings rooted/total number of cuttings tested) to compare the treatments (Payne, 2010). Analysis of variance was conducted and *t* tests were used to compare with the control using GenStat for Windows, Edition 16 (<http://www.genstat.com>). The 'Verdejo' clone was

used as a control, against which other varieties/clones were compared.

Results

SSTC method development. In Expt. 1, immature single-node cuttings formed roots 4 weeks after treatments (Table 2), but rooting rates differed. The best treatment was 70% methylated spirits and shaken for 30 s with the highest rooting percentage of 90% and relative low death and contamination rate. Rinsing with DI water had slightly lower rooting rate, higher death, and contamination rates than the 70% methylated spirits treatment although not significant at the 5% level. Seventy percent methylated spirits shaken for 30 s plus immersion in 0.5% liquid pool chlorine and shaken for 20 min did not alleviate contamination compared with the treatments of DI water or 70% methylated spirits. The cutting death rate was more than five times higher than treatment of DI water and more than eight times higher ($P < 0.05$) than that with 70% methylated spirits leading to a significantly ($P < 0.05$) lower rooting rate of 46.7% (Table 2).

Methylated spirits plus chlorine treatment was found to retard root formation with ≈ 1 week for the first rooting observed compared with the methylated spirits or DI water treatment (data not shown). Four weeks after putting onto rooting medium was long enough for root development in grape immature single-node cuttings. In the methylated spirits plus chlorine treatment, it needed 1 or 2 more weeks for root development in grape immature single-node cuttings before being transferred to a propagation bed. Root quality was similar in the treatments of DI water and methylated spirits, which was much better than in the methylated spirits plus chlorine treatment (Table 2).

The protocol with 70% methylated spirits and shaken for 30 s proved to be the best

procedure in establishing the SSTC method. The whole cycle of propagation (the period from an immature single-node cutting preparation to the point where rooted vines were ready for delivery) took 59 to 80 d. The procedure of the SSTC method is briefly described in Figure 2.

Comparison of rooting effect between cuttings propagation and SSTC method. The average rooting rate in the SSTC method was 82.5%, which was more than 16 times higher than in the cuttings propagation method (Table 3). The low rooting rate in the cuttings propagation was caused by rotting and death of immature single-node cuttings. Although a few immature single-node cuttings struck roots in the cuttings propagation, root quality was poor (Table 3; Fig. 3). In addition, the rooted immature single-node cuttings in the cuttings propagation method often were partially rotten at cutting ends. In comparison, the root quality of SSTC produced cuttings was robust as shown in Figure 3.

Application of SSTC method. The established SSTC method based on the 'Verdejo' clone was universally applied to all the genotypes tested in Expt. 3 (Table 4). Compared with the 'Verdejo' clone, all the other genotypes had similar ($P < 0.05$) rooting quality with rooting rates ranging from 80% to 93.8%. Contamination was low or non-existent.

The position in a vine where immature single-node cuttings was collected affected cuttings rooting performance. Most immature single-node cuttings from vine tips were dead and almost all nodal immature single-node cuttings survived.

Discussion

The most important aspect of a rapid propagation system is its ability to quickly provide relatively large amounts of planting stock to grape growers (Walker and Golino, 1999). The established SSTC method met

Table 2. Rooting performance of the 'Verdejo' clone immature single-node cuttings (4 weeks after treatment).

Treatments	Number of cuttings tested	Number of cuttings rooted	Root quality ^a	Number of cuttings dead	Number of cuttings contaminated	Means of cuttings rooted (%) $\pm$ SE	Means of cuttings death (%) $\pm$ SE	Means of cuttings contamination (%) $\pm$ SE
Deionized water (control)	34	28	5	3	3	82.4 $\pm$ 5.36	8.8 $\pm$ 0.43	8.8 $\pm$ 0.43
Methylated spirits	33	30	5	2	1	90.9 $\pm$ 4.54 NS	6.1 $\pm$ 0.26 NS	3.0 $\pm$ 0.09 NS
Methylated spirits + chlorine	45	21	1.3	23	1	46.7 $\pm$ 3.46**	51.1 $\pm$ 3.83***	2.2 $\pm$ 0.05 NS

^aGrade 1 to 5 from poor to good in terms of root number and lengths: 1 = started to root, 1 or 2 roots can be seen; 5 = sufficient quality roots are present for plants to be ready for nursery. A generalized linear model to the binomial data to compare the treatments. Approximate *t* tests were used to compare against the control using GenStat for Windows. The deionized water treatment was used as control, to which the other treatments were compared.

NS Nonsignificant; *significant at 5% level; **significant at 1% level; ***significant at 0.1% level.

Table 3. Rooting performance of 'Tempranillo' clone A immature single-node cuttings (3 weeks after treatment).

Treatments	Number of cuttings tested	Number of cuttings rooted	Root quality ^a	Number of cuttings dead	Means of cuttings rooted (%) $\pm$ SE	Means of cuttings death (%)
SSTC	40	33	5	7	82.5 $\pm$ 4.95	17.5
Cuttings propagation method	40	2	2.3	38	5 $\pm$ 0.17***	95

^aGrade 1 to 5 from poor to good in terms of root number and lengths: 1 = started to root, 1 or 2 roots can be seen; 5 = sufficient quality roots are present for plants to be ready for nursery. A generalized linear model to the binomial data to compare the treatments. Approximate *t* tests were used to compare against the control using GenStat for Windows. SSTC was used as a control, to which the cuttings propagation method was compared.

***Significant at 0.1% level.

SSTC = Semi-sterilized Tissue Culture.

this requirement and was established over a number of varieties as suitable for bulking up source materials quickly from limited mother vines.

It only took ≈ 2 months from the cuttings preparation to availability to the propagators/growers in the SSTC method as shown in

Figure 2, whereas it takes 3 months (Singh et al., 2004) to 6 months (Mhatre et al., 2000) in reported micropropagation techniques involving explant initiation to the point ready for transferring planting out in the field. The immature single-node cuttings were much thicker than used in vitro microcuttings from

comparisons with previous experience using micropropagation techniques on grapevines, giving the immature single-node cuttings more accumulated carbohydrates available for use in root strike and growth processes (Gordon, 2009; Read, 2007). As a result, this may have allowed the immature single-node cuttings to generate roots more easily taking less time to grow to the desired size as shown in Figure 1. Significant time was also saved in the SSTC method by excluding explants' initiation, in vitro establishment, and proliferation, which are mandatory in the micropropagation method.

In the SSTC method, the sterilization requirement was not as strict as in micropropagation, which made this method easy to operate. In micropropagation, 100% of sterilization of cuttings is required, whereas in the SSTC method, the explants (immature one-node cuttings) did not have to be 100% sterilized, which appeared to have little effect on the cuttings' ability to root because of the short time in culture. There was also the added advantage that single nodal cuttings were healthier as a result of not suffering damage from chlorine as used in stricter sterilization procedures. In the SSTC method, the sterilization needed to be sufficient to remove pathogens but not too severe to damage tissues causing rotting and death or affecting root formation in immature cuttings. If sterilization was too light, contamination of explants would be severe resulting in a lower successful rate as a result of explants being destroyed such as in the DI water treatment in Expt. 1. If too harsh, explants would die resulting in lower rooting rate such as in the treatment of methylated spirits plus chlorine in Expt. 1 (Table 2). In addition, the treatment that included a chlorine solution retarded root formation. The contamination rate in Expt. 1 was generally low. There was no significant improvement in the treatment with methylated spirits plus chlorine compared with the treatment with methylated spirits only in Expt. 1, indicating that treatment with bleach was not necessary in controlling contamination in the SSTC method. Immature one-node cuttings used in the SSTC method were sourced from potted plants in the glasshouse where the pathogen population was generally low. Light sterilization was sufficient, having the advantage of producing robust cuttings growth and rooting ability. If the cuttings were to be sourced from the plants in an open field, a harsh sterilization procedure would be considered as a result of their exposure to more potential pathogens.

The SSTC procedure developed simplifies propagation by removal of explant initiation, in vitro establishment, multiplication, and maintenance like in the micropropagation method. This is less demanding of high tissue culture expertise and skills. In addition, the new technique avoided the degeneration and bacterial contamination problems occurring in the tissue culture process because explant initiation and maintenance were no longer required. The simplified technique was less expensive and

Fig. 1. Seedlings of 'Tempranillo' clone A ready for delivery, propagated by Semi-sterilized Tissue Culture (SSTC).

Fig. 2. Diagram of stages of the Semi-sterilized Tissue Culture (SSTC) propagation process (number of days listed applies to the spring and summer seasons in Perth, Australia).

Fig. 3. Rooting performance of 'Tempranillo' clone A in Semi-sterilized Tissue Culture (SSTC) and comparison with cuttings propagation method. (A) Rooted immature single-node cuttings 21 d after treatment in the SSTC method; (B) rooted and dead immature single-node cuttings 21 d after planting in propagation potting mix in the cuttings propagation method.

easy to adopt as a commercial propagation approach.

Immature single-node cuttings were used for propagation in the SSTC method, which improved cutting yield from cutting material. In the mist propagation, herbaceous cuttings with at least two nodes and two lateral buds had to be used (Walker and Golino, 1999). This efficiency was critical when source material was limited such as for grapevines released from quarantine. Because immature single-node cuttings failed to root and were of poor quality, the cuttings propagation method could not be effectively used. One of the possible reasons why the tender immature single-node cuttings failed was because they could not tolerate the high moisture in the potting mix and high humidity, which has been found to have an important role in green cuttings propagation when using herbaceous cuttings such as in mist propagation (Walker and Golino, 1999).

Micropropagation is a tried and tested approach to achieve a high multiplication rate in some vines. To achieve this, addition of cytokinins to the culture medium was essential for culture initiation (Singh et al., 2004) and for the sprouting and formation of multiple shoots (Ibáñez et al., 2003) in vinifera cultivars. Good proliferation rates were achieved by applying cytokinins at high concentrations ranging from 5 to 17.75 µM 6-benzylamino purine (BAP) or 6-benzyladenine (Gray and Fisher, 1985; Heloir et al., 1997; Ibáñez et al., 2003; Mhatre et al., 2000; Singh et al., 2004). During micropropagation of 'Pinot Gris', supplementation of 5 µM BAP led to two to three shoots generated per explant per subculture. However, further transfers beyond two to three subcultures induced vitrification and degeneration of the in vitro explants (data not shown). This has also been reported by other researchers (Heloir et al., 1997; Ibáñez et al., 2003). The SSTC method is advantageous because it minimizes the in vitro handling procedure and avoids the problems associated with tissue culture. Good root quality is important for microcuttings survival after being deflasked (de Fossard, 1981) and poor rooting is a major obstacle in micropropagation (De Klerk, 2002). Poor rooting causes losses at

the acclimatization stage, which reduces output making the product too expensive to be commercially viable (Barlass and Hutchinson, 1996; Simmonds, 1983). The roots formed using the SSTC method were robust and already established in the propagation medium with less than 5% loss of rooted vines during acclimatization.

During this research we found the stage of shoot growth of source material was important and that shoot tips at the top 2 to 5 cm of a shoot were too immature for this new method. Similarly, cuttings from the most succulent tissues often failed to root in mist propagation of young cuttings (Walker and Golino, 1999). This might be caused by a low carbon:nitrogen (C:N) ratio, which has been found in other plants (Brandon, 1939; Kraus and Kraybill, 1918; Starring, 1924). Shoot tips with the fastest growth rate would be rich in nitrogen resulting in low C:N ratio, which might have led to their rooting difficulty. The high moisture environments might cause their rotting and death. Although cuttings that are high in stored carbohydrates have the capacity to develop good root systems (Gordon, 2009), cuttings collected from shoots of source plants undergoing active growth may have been low in carbohydrates and therefore root production was poor. The C:N ratio is just one of the many factors that influence success in plant propagation. There are many others such as proactive control of petri diseases, choice of optimum source materials, and application of correct practices (Fourie and Halleen, 2004, 2006; Gordon, 2009).

There are some limitations in the SSTC method because it relies on the generation and growth of new shoots, which are affected by seasons. Propagation using SSTC could not be done in winter when the source plant went dormant. Placing the source plants into a warm glasshouse did not greatly extend the growth time to make immature cuttings available for propagation. The micropropagation method, supposedly allowing all year-round production as an advantage, is also limited by seasonality in explants as found for barley (Sharma et al., 2005). Our experience also showed that it was difficult to maintain normal growth and multiplication of grapevine microcuttings in vitro in winter when the

vines normally went dormant in open fields, and this seasonality problem was not overcome by tissue culture. It seemed that the built-in biological clock was still controlling plants' development and able to switch vegetative and reproductive modes on and off when the temperature and daylength (photo-period) were constant under tissue culture conditions at least in some plant species, if not all. This is supported by evidence from other crops. For instance, in another study (unpublished data), *Dampiera* sp., an Australian native plant, also had a seasonal response, although they had been maintained in vitro for several years. The reproductive development initiated at the similar date as in nature with flowering occurred in vitro if the flower buds were not removed. This led to little growth, no multiplication, and easy death of shoots after flowering. It suggests that the circadian rhythms (Yakir et al., 2007) remember seasons and this can disrupt tissue culture multiplication and explant growth.

In summary, a novel semisterilization method using immature single-node cuttings, SSTC, was developed to allow rapid propagation of grapevines (*Vitis vinifera* L.). SSTC was more efficient with a 16 times higher root strike rate than the cuttings propagation method and took at least 50% less time than the micropropagation method to produce robust planting stocks. The propagation procedure in the SSTC method engaged a shorter time in culture as a result of removal of explant initiation and in vitro explant establishment/proliferation/maintenance processes than in micropropagation methods. It allowed use of much less robust and smaller cuttings than traditional cutting methods maximizing yield from limited source material. The demand on tissue culture expertise and skill was not as critical any more as in the micropropagation method. In addition, the new technique avoided the degeneration and bacterial contamination problems occurring in the tissue culture process. The simplified procedure was less expensive and easy to be adopted as a commercial propagation approach. This technique was also tested successfully on cassava with a rooting rate at almost 100% (unpublished)

Table 4. Rooting performance of grape varieties/clones using Semi-sterilized Tissue Culture (4 weeks after treatment).

Variety/clone	Number of cuttings tested	Number of cuttings rooted	Root quality ^a	Number of cuttings dead	Number of cuttings contaminated	Means of cuttings rooted (%) ± SE	Means of cuttings death and contamination (%)
'Verdejo' clone (control)	85	73	5	12	1	85.9 ± 3.26	14.1
'Pinot Gris' clone	80	72	5	8	0	90.0 ± 2.97 NS	10.0
'Pinot Noir' clone	83	70	5	12	1	84.3 ± 3.37 NS	14.5
'Tempranillo' clone A	96	78	5	17	1	81.3 ± 3.25 NS	17.7
'Tempranillo' clone B	128	109	5	19	0	85.2 ± 2.64 NS	14.8
'Tempranillo' clone C	96	86	5	7	3	89.6 ± 2.78 NS	7.3
'Tempranillo' clone C	95	88	5	7	0	92.6 ± 2.50 NS	7.4
'Tempranillo' clone E	70	56	5	12	2	80.0 ± 3.84 NS	17.1
'Merlot' clone	30	27	5	3	0	90.0 ± 4.95 NS	10.0
'Savagnin' clone	124	101	5	23	0	81.5 ± 2.85 NS	18.5
Total	887	760		120	8	85.7	13.5

^aGrade 1 to 5 from poor to good in terms of root number and lengths: 1 = started to root, 1 or 2 roots can be seen; 5 = sufficient quality roots are present for plants to be ready for nursery. A generalized linear model to the binomial data to compare the treatments. Approximate *t* tests were used to compare against the control using GenStat for Windows. The performance of the 'Verdejo' clone was used as control, to which the performance of other varieties/clones was compared.

NS Nonsignificant; *significant at 5% level; **significant at 1% level; ***significant at 0.1% level.

indicating this method could be transferred to other crops.

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