TISSUE CULTURE PROPAGATION OF KIWIFRUIT

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Abstract. Since kiwifruit cultivation is increasing rapidly in Italy, better propagation methods are needed. A method is described for tissue culture propagation of kiwifruit by meristem-tips dissected from resting buds and actively growing shoot tips using a modified Lepoivre medium A multiplication rate of 5 3 was achieved for each 30-day subculture period Single shoots, 30 to 35 mm long, with 2 to 4 leaves, could be rooted in paperpots containing a soil-like mixture, wetted by half-strength macro-micro nutrient solution After 3 weeks, about 90% of shoots rooted and within 60 days all developed into plants 150 to 200 mm long with 6 to 10 leaves.

Kiwifruit (*Actinidia chinensis* Planch.) is a relatively new and increasingly important commercial crop in Italy. In 1978 the cultivated area was only 800 hectares, but this had increased to 1,860 hectares by 1983. Of this area, 816 hectares were in production with the remainder in young, non-bearing plantings. The distribution of the plantings is 62% in the north (Po Valley and near the French and Austrian borders), 30% in central Italy (near Rome) and 8% in the south and on Sicily and Sardinia. Most plantings (72%) are less than 1 hectare; the average area per planting is larger in central Italy and in the southern part than in the northern part (16).

'Hayward' is the leading pistillate cultivar (72%) with 'Abbott' and 'Monty' the other major ones. The staminate cultivar generally used is 'Tomuri', but in more than 50% of the orchards the pollenizer is unknown. Drip or spray irrigation is used on 81% of the farms. For new orchards, plantlets from self-rooted cuttings and/or from grafting on seedlings are usually employed.

These vegetative propagation techniques require 2 to 3 years in order to obtain commercial plants. Furthermore the traditional propagation methods are not very productive. Consequently the possibility of propagating kiwifruit by *in vitro* techniques was tested. Several preliminary trials on kiwi propagation *in vitro* have been carried out (2, 5, 6, 7), but these methods were not fully effective for commercial applications.

In the Fruit Science Institute of the University of Perugia, several investigations have been carried out since 1979 regarding the micropropagation of 'Hayward' and 'Tomuri' kiwifruit (11, 12, 13, 14). In this report we describe the stages of kiwi micropropagation indicating that this technique can be employed as an alternative method of vegetative multiplication.

METHODS AND RESULTS

Stage 1. Choice of initial explant and establishment of the aseptic culture.

As initial explant we have used both meristem-tips (0.2 to 0.5 mm long), and apical shoots (10 to 15 mm long), because presumably they have the highest genetic stability (1, 4, 15). The woody shoots with buds were collected during the winter, sprayed with benomyl (0.3 g/liter), and then stored at 1°C.

From these buds the meristem-tips were excised during March and April, using the following sterilization method, the best among several tested. Initially the woody shoots were dipped for 5 minutes in ethanol (80% v/v), soaked for 20 minutes in a solution of 4% calcium hypochlorite and rinsed twice in sterile distilled water. The meristem-tips were dissected aseptically under a stereo microscope.

Single meristem-tips were placed in vials (15 × 100 mm) containing 4 ml of the medium listed in Table 1. These cultures were placed in the dark overnight and then transferred to a growth room at 24 ± 1°C and 2.1 klux of light provided by Philips TL 40W/33 and Sylvania Grolux fluorescent lights. Light was provided for 1 hour out of every 3 the first day increasing to 16 hr light and 8 hr dark after 15 days.

We preferred using meristem-tips rather than apical shoots as initial explants because the dormant buds (a) can be stored at low temperatures extending the time at which cultures can be initiated, (b) can be sterilized more easily than herbaceous shoots, and (c) can produce virus-free plants if very small (0.2 to 0.3 mm) meristem-tips are taken. However, the dissection of meristem-tips requires more time and care than apical shoots and the meristem-tips take longer before producing the first shoot.

Within one week of the beginning of the culture, 30 to 40% of meristem-tips are usually contaminated, but this value rises to 60 to 70% when apical shoots ar employed as initial explants. After 20 to 40 days, meristem-tips developed into rosettes 5 to 10 mm high with 2 to 4 leaves. Increasing naphthaleneacetic acid (NAA) levels in the establishment medium resulted in callus formation at the base of the rosette. Indole-3-acetic acid (IAA) at 0.005 and 0.05 mg/liter was no better than NAA, but produced callus at 0.5 mg/liter.

Table 1. Composition of organization, proliferation and rooting-acclimatization media

COMPOUNDS	STAGE 1 mg/l	STAGE 2 mg/l	STAGE 3 mg/l
MACROELEMENTS			
KNO_3	1,800	1,800	900
$Ca(NO_3)_2$ $4H_2O$	1,200	1,200	600
NH_4NO_3	400	400	200
$MgSO_4$ $7H_2O$	360	360	180
KH_2PO_4	270	270	135
MICROELEMENTS			
$FeSO_4$	27.85	27.85	27.85
NaEDTA	37 25	37 25	37 25
$ZnSO_4$ $7H_2O$	10	8.6	8 6
H_3BO_3	10	6 2	6 2
$MnSO_4$ H_2O	18	1 0	0.025
$CuSO_4$ $5H_2O$	0 025	0 025	0.025
Na_2MoO_4 $2H_2O$	0.25	0.25	0 25
KI	0	0.08	0 08
$CoCl_2$	0	0.025	0 025
ORGANIC COMPOUNDS			
Myo-Inositol	100	100	100
Nicotinic acid	5	0	0
Glycine	2	0	0
Pyridoxine HCl	0.5	0	0
Thiamin HCl	0.5	4	4
Folic acid	0.5	0	0
Biotin	0 65	0	0
PHYTOHORMONES			
Gibberellic acid* (GA_3)	0 1	1 0	0
Benzyladenine (BA)	0 5	1.0	0
Naphthaleneacetic acid (NAA)	0 02	0	0
Sugar	20,000	20,000	0
Agar	7,000	7,000	0
pH**	5 8	5.5	5 5

All the media were autoclaved at 110°C for 25 and 30 minutes (stages 1, 2 and 3, respectively)
* Added after autoclaving by millipore filter (micron 0 22).
** Adjusted by 0.1 N NaOH in agar-media and by $CaCO_3$ in soils

Stage 2. Proliferation.

For the first subculture 25 × 160 mm vials containing 20 ml of proliferation medium (Table 1) were used. Culture conditions were 24 ± 1°C with a photoperiod of 16 hr and light intensity of 2.1 klux from fluorescent lights.

After 20 to 30 days each rosette developed into one or two shoots, 10 to 20 mm long, with 2 to 4 leaves (Figure 1). Five of these shoots were placed into 500 ml jars containing 200 ml of medium (Table 1). After 30 days in this proliferation medium, in the same environmental conditions mentioned above, each shoot produced an average of 5.3 new shoots. Attempts to

increase the multiplication rate were carried out by testing 15 macro- and micronutrient combinations, selected among media formulations suggested by other authors (3, 8, 9, 10) for several woody plants. However, the medium formulation listed in Table 1 showed the best proliferative activity. Some nutrient conditions caused a higher multiplication rate, but the resulting shoots did not root and/or showed a large callus at the base.

Four different concentrations (0, 0.1, 1, 10 mg/liter) of both gibberellic acid (GA_3) and benzyladenine (BA) combinations were tested without mineral nutrient modifications. The best results were obtained when both growth regulators were present at 1 mg/liter (Table 2).

Figure 1. Kiwifruit shoot developed from rosette after 3 weeks on proliferation medium.

Table 2. Multiplication rates observed in the various hormonal combinations.

BA concentration (mg/liter)	GA_3 concentration (mg/liter)				
	0.00	0.01	0.10	1.00	Mean
0.00	1.3	1.6	1.4	1.6	1.5
0.10	3.5	2.3	2.9	2.8	2.9
1.00	3.6	4.0	3.9	5.0	4.1
10.0	1.8	2.0	1.5	1.5	1.7
Mean	2.6	2.5	2.4	2.7	—

GA_3: Gibberellic acid.
BA: Benzyladenine.

Stage 3. Rooting and acclimatization.

For rooting purposes shoots 30 to 35 mm long with 2 to 4 leaves were used. Culture conditions were the same as for Stage 2. Initially 5 shoots were placed in each jar containing 200 ml of rooting medium (Table 1) with indole-3-butyric acid (IBA) at 1 mg/liter. With this treatment, only 37.5% of the cuttings rooted and the new plantlets had a large basal callus. The rooting percentage improved to 75% when the shoots were dipped in sterile solution of 10 mg/liter IBA for 5 seconds and then placed in agar medium (Table 1) without auxin. When the shoots were placed for 10 days in a medium with 1 mg/liter IBA and then transferred to auxin-free medium, 80% rooting was obtained. After 4 weeks, these plantlets were transplanted into pots containing a peat-sand mixture (1:1) and left in the growth room under a plastic tunnel with decreasing humidity for 10 days.

Then the pots containing the plantlets were transferred to a greenhouse under intermittent mist. Although rooting had been satisfactory, many of the plantlets died during this step.

In an attempt to overcome this problem, the following method was tested. After proliferation, single shoots were placed in paperpots (38 × 35 mm) containing a soil-like substrate (Torboflor) wetted by Stage 3 macro and micro elements (Table 1) and with a pH level of 5.5 adjusted by powdered marble ($CaCO_3$). Then, 20 paperpots were placed in closed glass jars, (Figure 2) sealed by Parafilm and transferred to growth room conditions. After about 3 weeks, the emergence of physiologically functional roots occurred. This was detected by the growth restart of plantlets (Figure 3). At this time the jar caps were removed and containers were left in the same growth room and the plantlets were sprayed periodically with water. After 2 weeks, the plantlets were transplanted to pots containing Torboflor, without disturbing the root systems, and then transferred into a greenhouse. This method avoided root system acclimatization stress. As a consequence, about 90% of the shoots rooted and developed into plantlets that reached heights between 15 and 20 cm with 6 to 10 leaves in 2 months.

OTHER OBSERVATIONS

Shoots of 'Hayward' kiwifruit subcultured monthly for a period of over two years and those subcultured only 3 to 4 times were compared. Increase in the number of subcultures was accompanied by an increase in multiplication rate and a progressive decrease in shoot length and callus weight at the base of the explant. The rooting ability of shoots and the number of chromosomes found in the root apex remained

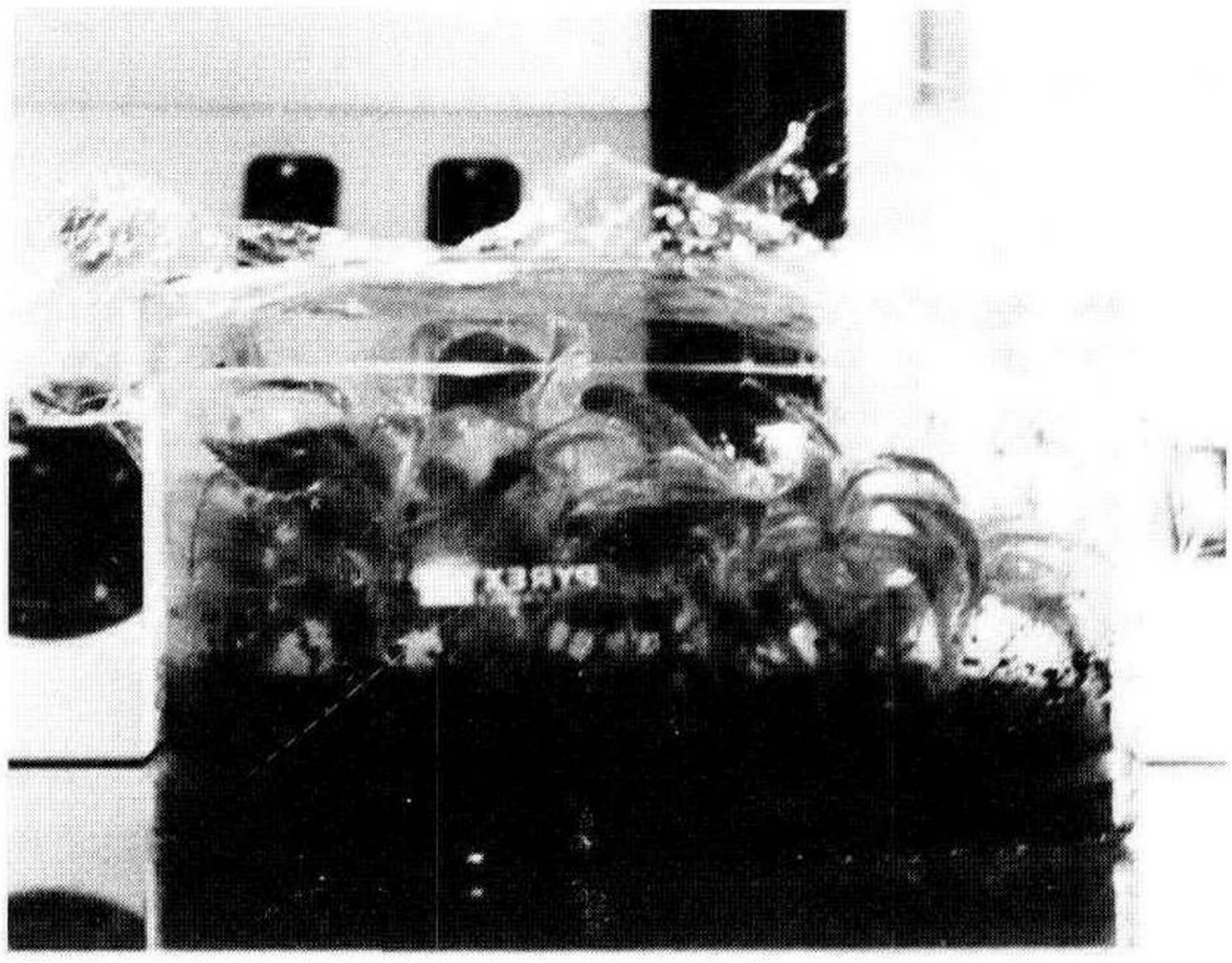

Figure 2. Shoots being rooted in "Torboflor" contained in 38 × 35 mm paper pots placed in large, closed glass container.

Figure 3. Rooted plants in paper pots ready to be transplanted to larger containers 5 weeks after beginning of rooting.

constant. The following mean values were measured after the third subculture: a) multiplication rate, 3.4; b) shoot length, 4.4 cm; and c) callus weight, 0.50 g; while after 28 subcultures these were 5.8, 3.5 cm and 0.23 g, respectively. Similar results were found with explants from the 4th and 29th subcultures and they also showed no correlation between multiplication rate and shoot length. There was no significant difference in rooting ability in shoots obtained at different culture ages, except for the average number of roots per rooted plantlet, which was higher in plantlets obtained after the 28th subculture (Table 3).

Table 3. Rooting ability of shoots after 3 and 28 subcultures

Number of subcultures	Means of 50 shoots*		
	Rooting (percent)	roots/plant (number)	root/length (mm)
3	88.0 a	3 8 a	35 a
28	96 0 a	5 6 b	31 a

* Mean separation within columns by Duncan's multiple range test, 5% level

It seems, therefore, that kiwifruit shoots have good genetic stability and do not lose their morphogenetic capacity, even when kept for a long period of time in proliferation phase in the conditions described here. The increase in multiplication rate and decrease in callus weight that accompanied an increased number of subcultures show that 'Hayward' is well adapted to *in vitro* culture conditions. Finally these satisfactory results permit us to believe that kiwifruit can be vegetatively propagated by the *in vitro* culture technique. Nevertheless, behavior in the field of micropropagated plants must be checked and compared with that of plants originating from traditional methods of multiplication. For this purpose, in 1983, micropropagated plants, and those from self-rooted cuttings, were planted in the field and are being observed to evaluate their field performance and phenotypic stability.

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