Propagation of Fruit Trees by Tissue Culture in Italy

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Clonal propagation of fruit trees and other plant species through tissue culture is based on the concept of cell totipotency first expressed by Virchow in 1858 (54) which derived from the cell theory that Schleiden proposed in 1838 (46). Technical problems made it impossible to put these ideas into practice, and more than a century passed before plants could be cultured in vitro. Plant tissue culture developed with the improvement of technology; thus, it has not been a revolution in theory but an evolution of techniques. The origins of plant tissue culture trace to studies by Haberlandt (14), who aseptically cultured single cells on a liquid medium supplemented with sugar. The cells remained alive, but were unable to proliferate.

The identification and purification of 1*H*-indole-3-acetic acid (IAA), the first known growth regulator, by Kogl et al. (25) in 1934 and then by Thimann (51), made it possible to control the growth of plants, tissues, and cells. In 1939, Gautheret (12) (and also Nobecourt) cultured carrot callus for an indefinite time, but almost 20 years had to pass before morphogenesis was achieved. Reinert (40) and Steward and Mapes (48) were the first to obtain plantlets from callus and cell-suspension cultures. This success was achieved using medium containing inorganic salts, sugar, amino acids, vitamins, and some undefined organics such as malt extract and coconut water. The use of undefined substances caused some problems, because it was not possible to quantify their contribution. An enormous literature on plant tissue culture now exists (64). This review will be devoted to recent results on the in vitro propagation of temperate and subtropical fruit crops of importance in Italy.

METHODS FOR IN VITRO PROPAGATION OF FRUIT TREES

In the past 20 years, tissue culture has become a useful research tool for botanists, cytologists, geneticists, and pathologists, and has also provided a substantial applied contribution to many fields, from industrial applications such as metabolite production for pharmaceuticals to propagation for nursery production. The initial impact of tissue culture on the nursery industry dates to 1964, when Morel (34) clonally propagated orchids from shoot tips. Subsequent studies can be traced back to this research, and great progress has been achieved with other horticultural species.

Research on fruit trees developed later, but so much work has been accomplished that, within a few years, it has been possible to move from laboratory studies to commerical nursery production. Italian scientists and nursery workers have played a role in this technology and their contribution will be stressed in this review.

There are three reasons why commerical propagators use micropropagation for fruit tree production: a) the possibility to begin with a very small number of plants (even only one); b) it is possible to multiply pathogen-free plants in a short time and in a small space; and c) it is possible to adjust the rate of production according to the nursery or marketing needs, since in vitro-cultured propagules do not depend on an in vivo vegetative cycle. There are at present three techniques of in vitro propagation—adventitious meristem formation, somatic embryogenesis, and axillary shoot multiplication.

Adventitious meristem formation (organogenesis)

The initiation of meristematic activity for shoot and/or root production has been described (13, 52, 53) and the steps are well-known. These meristems probably originate from a small group of cells that can be either superficial or situated inside the callus.

The induction of morphogenesis is often controlled by the cytokinin : auxin ratio of the basal medium (45). To obtain shoot formation, a high cytokinin concentration is required, while a high auxin level promotes the induction of root primordia. Generally, when using this technique for propagation, shoot formation is induced first and then the shoots (microcuttings) are detached and rooted in a different medium. This method for example, is used for kiwifruit, grapes, *Prunus* spp., and *Malus* spp. (1–3, 10, 15–17, 23, 26, 28, 43, 44, 62–64). Although growth regulators are responsible for the induction of organogenesis, this process is also under the control of physical, nutritional, and physiological (origin of callus) factors (55). In theory, it is possible to propagate an enormous number of plants from a single meristem. Application is limited by difficulties in the control of adventitious meristem formation in several woody species or specific clones within the species, and because of the risk of obtaining aneuploid, polyploid, or aberrant plants.

Somatic embryogenesis

Somatic embryogenesis is a natural phenomenon in several fruit tree species (e.g., *Citrus, Mangifera, Malus,* and *Juglans*) and occurs as an alternative (apomixis) to or together with sexual reproduction (polyembryony). Hence, it is often difficult to differentiate zygotic and somatic embryos.

Reinert (40) and Steward and Mapes (48) were the first to obtain somatic embryos in vitro from culture of secondary phloem of carrot roots. This finding has been observed subsequently in many angiosperms. Somatic embryos also have been produced from tissues of mature and immature embryos as well as petioles, leaves, roots, flower axils, anthers, mesophyll cells, and protoplasts (55). These embryos originate by several mitotic divisions, generally starting from a single cell on the callus surface or from the periphery of cell clumps in liquid culture.

Growth regulators are involved in somatic embryogenesis but their action is still unclear. It seems that auxins (2,4-D in particular) are required to induce embryogenesis, but this substance is not necessary and may even have an inhibitory effect on embryo development (55). Embryo induction usually requires an auxin-rich medium and embryo development requires an auxin-poor medium. In vitro-induced embryogenesis has a great potential for the species that respond to this technique, because it is possible to obtain clonal production of plants rapidly. This clonal production has been achieved for an increasing number of species. In vitro somatic embryogenesis has been reported in many fruit crop species including citrus, cocao, apple, strawberry, papaya, and oil palm, and is being used for oil palm propagation in Africa (30).

Axillary shoot multiplication

This technique is based on the in vitro formation of new vegetative axes and buds produced in the axils of young leaves or leaf

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primordia, starting from a single bud or already differentiated meristem. At the beginning of the culture, this method is less efficient than organogenesis or embryogenesis but, after frequent subcultures, the rate of multiplication may increase greatly.

This increase in the proliferation rate seems to depend on the process of rejuvenation that occurs during culture. Also, positive results obtained with the use of 1,3,5-trihydroxybenzene [phloroglucinol (PG)] in the nutrient medium seem to be related to the rejuvenation process; in fact, the positive effect of PG is greater in cultures that are initiated from older stock plants. The rejuvenation process also has a positive influence on the rooting phase, increasing the percentage of rooted shoots. Most information on this process relates to apple trees (22). For instance, in the culture of 'Golden Delicious' apple, proliferation after the third subculture exceeds a rate of 20 to 1. Subculturing for a whole year, in theory, could produce 4×10^{15} plantlets. The number of plantlets produced is remarkably high, even considering one-half of this potential and culturing for a shorter time period.

It has not been possible in commercial nursery production to achieve these results, but, considering the species, the number of plants produced is still remarkable (Table 1) (31).

Axillary shoot multiplication consists of several distinct phases: a) establishment in culture of pathogen-free buds; b) proliferation (multiplication of axillary buds through the formation of new vegetative axes); c) conditioning and elongation of miniature shoots; d) rooting; and e) transplanting and establishment in vivo. The first phase involves disinfestation to establish aseptic cultures; elimination of viruses, fungi, and bacteria requires special techniques (62). Greenhouse-grown stock plants are preferred because almost 100% of field-grown material is infected. Next, the sterile bud is recultured on a new medium that contains growth regulators, including a cytokinin to break apical dominance. After a period of time varying from 2 to 8 weeks (depending on the species), miniature shoots form and axillary buds break and immediately develop shoots.

The number of new axes that are formed from one explant increases until the third or fourth subculture and then tends to stabilize. This process, in theory, could be repeated indefinitely, but, for reasons still unknown, the material sometimes appears to age and become less responsive. The problem of genetic stability and of production of true-to-type plants must be kept in mind; it is better to limit the number of subcultures to avoid this risk. Electrophoretic "finger-printing" can be applied to assess trueness-to-type of plants obtained from prolonged cultures or from callus (56).

The control of morphogenesis during the multiplication phase to achieve early budbreak is moderated by cytokinins. Typically, *N*-(phenylmethyl)-1*H*-purin-6-amine (BA) is applied in concentrations ranging from 1 to 25×10^{-6} M. The next step involves induction of root primordia (rooting phase). Single miniature shoots (1–3 cm) of three to 10 internodes are rooted in a medium different from the proliferation medium. Growth regulators involved in this process are auxins. For improved control, a transition phase lasting about 2 weeks is necessary, during which time the shoots can elongate to allow easier handling. The material can also be conditioned during this time; for example, interruption of photoperiod for a few days before rooting seems to have a positive effect (19). Conditioning reduces the negative effect of cytokinins on root formation and hardens the plantlets, a process important after transplanting.

Auxins can be applied within the agar medium in which the shoots are planted, or these can be handled as microcuttings by dipping the shoot ends for a short time in a concentrated solution and then planting them in a nutritional medium without growth regulators, or even directly in vivo. Direct planting, which significantly lowers production costs, has given positive results with several species such as apple (42), kiwifruit (3, 46, 47), and rhododendron (61). Transplanting and acclimation is a critical phase in the process.

Recent research conducted on several species has pointed out that leaves of tissue-cultured plants often show some structural changes compared to plants grown in vivo. Changes are involved in mesophyll tissues (6, 59), the amount of epicuticular waxes (11, 60), and the stomata (5). The efficiency of stomata seems to be altered to reduce water-loss resistance of the plantlets during acclimation (6, 7).

The transfer to in vivo conditions usually follows a very simple procedure. At the proper time, the plantlets are treated with fungicides and transferred from the jar or test tube to containers filled with previously sterilized peat and sand (or other materials) and then placed under controlled conditions. It is important to maintain high relative humidity and low light intensity. Callusing of the shoot end during the rooting phase must be avoided. More investigations are required on this aspect.

Table 1. Estimated proliferation potential for some fruit species, starting from 100 sterile buds (data from Z. Vivai, personal communication).

Culture period (weeks)	Fruit			
	Kiwi	Apple	INRA GF677	Pixy
0	0.1	0.1	0.1	0.1
4	0.2	0.3	0.4	0.5
7	0.4	0.9	1.6	2.5
10	0.8	2.7	6.4	12.5
13	1.6	8.1	25.6	62.5
16	3.2	24.3	102.4	312.4
19	6.4	72.9	409.6	1562.5

PROBLEMS WITH IN VITRO MASS PROPAGATION OF FRUIT TREES BY MULTIPLICATION OF AXILLARY BUDS

Choice of material

A number of investigations conducted for many years on several species (27) has indicated that increased efficiency of in vitro mass propagation requires not only selection of the proper organs and tissues but also the use of an appropriate phenological stage. For all fruit crops tested, young, actively growing parts of the plant show the highest morphogenetic and regenerative capacity (P. Fiorino and A.R. Leva, unpublished data).

When it is either desired or necessary to start from dormant organs, these must be isolated at the initial stage of their development. In vitro regenerative capacity seems to be associated with biological activity controlled by endogenous factors — these factors are different depending on the phenological stage of the plant — thus, control with exogenous growth regulators is limited.

Most organs or tissues growing in culture tend not to show a cyclic behavior; however, research should be conducted with a greater variety and number of samples because some data obtained from tests on the rooting ability of apple suggest a cyclic response in vitro (A.R. Leva, unpublished data).

Disinfestation of explants

In some cases, disinfestation can be a critical point in the establishment of certain species or cultivars in culture. Field-grown material of many species cannot be used. Shoots from hardwood cuttings in the laboratory are the most practical solution in many cases. The importance of the phenological stage indicates that there is a particular time of the year for every species, depending on the organ, species, and clone that is optimum for explantation. Disinfestation is an obligatory step, whatever the level of infection. Generally, the most useful sterilizing agents are NaClO, $CaCl_2O_2$, H_2O_2, or ethanol. All of these treatments may be used either separately or in mixtures (except H_2O_2). Addition of a wetting agent allows better contact of the solution with the plant surface. A 10-min dip in the sterilizing solution is generally sufficient (a few seconds with ethanol from 70% to 90%). Vacuum sterilizing may be helpful with some species particularly difficult to sterilize, but tissue damage increases. The use of antibiotics and fungicides has given successful results (50, 62), but extensive use has been limited.

In order to secure healthy buds for explanting, fruit trees must be grown in pots or under controlled conditions, irrigated by drip system, and treated so as to reduce infestation. It is also important to handle the material carefully from the moment it is dissected from the plant until disinfestation.

Cultural environment

The in vitro culture environment is usually a growth chamber to control temperature and light, including photoperiod, intensity, and wave length. A photoperiod of 16 hr of daylight is usually optimum, but a longer period of darkness may be beneficial for rooting. Attention must be paid to the latter aspect. In fact, with 'Rougeon' grapes, best results have been obtained under 10-hr photoperiod as compared to continuous light or 15-hr photoperiod and darkness (8). Few studies have been conducted on the influence of light intensity and frequency in fruit tree propagation. Illuminance usually ranges from 3 to 5 klx. Satisfactory results have been obtained with cool-white florescent lights, including Grolux. A relevant interaction between light intensity and wavelength that affects proliferation rate and growth of explants has been pointed out. The yellow-red band (570 nm) seems to have a positive effect, while the near-ultraviolet (320–380 nm) has a negative influence. Optimum temperatures range from 20° to 28°C, but little research information is available.

Particular attention must be given to the atmosphere. It has been hypothesized (35) that some polluting agents, such as CO_2, ethylene, or ethanol, have a negative effect on morphogenesis. These gases (other than CO_2) are not usually present in the atmosphere, but, in large micropropagation laboratories, gas levels (especially alcohols, ethers, and esters) can become biologically important. Even the size of the culture vessels can have an influence on the proliferation phase (33).

Culture medium

The components of the culture medium can be divided into four groups: inorganic salts; organic compounds that are chemically defined (such as sugars, vitamins, amino acids, and growth regulators); undefined organic compounds (such as malt extracts, coconut water, and fruit juices); and a gelling material for semi-solid medium, which should provide a physical support to the explant without interference with growth. Since there is physical activity, interference with solute movements can create diffusion gradients in the medium. Gelling materials should not have any active or relevant chemical role, although some inhibitory effect has been found (26).

There are now many basic media available that were developed to satisfy the nutritional requirements of explants. The most common is the MS medium of Murashige and Skoog (36). This medium has undergone many variations and improvements, but the great majority of formulations now employed are derivative and MS is still the medium of choice in most instances. The basal medium must include: N, both in the nitrate and ammoniacal form; K, Ca, Mg, P, and S in their most oxidized forms; and a number of microelements; and, in addition, some micronutrients are present as impurities in the forms of major elements used.

Three organic substances are fundamental: sugar (sucrose), which represents the best carbon and energy source; thiamin, the only vitamin that is absolutely necessary for all species tested, and myo-inositol, a vitamin that may be essential for woody species. Recently, Ishihara and Katano (18) reported that these two substances were ineffective with an apple cultivar.

In the past, the use of undefined compounds was of some use. These substances, mainly mixtures, have an indefinite and variable composition (e.g., coconut water is the liquid endosperm of the enlarging fruit). The use of these compounds has been almost completely abandoned because it is impossible to identify the factors responsible for morphogenic processes in these mixtures.

Agar is commonly employed to gel the medium, although particular advantages have sometimes been found with pectins that seem to allow better contact between the explant and the medium, especially when explants are very small. Sometimes it is not necessary to employ a semisolid medium. In *Prunus,* for example, the rooting phase is more efficient in a liquid medium with a paper bridge (4, 15).

Antibrowning and absorbing compounds added to the medium are often fundamental for survival during the first and sometimes the second subculture. In vitro establishment of some species (*Castanea sativa* Mill., *Juglans regia* L., *Malus pumila* Mill, and *Hamamelis* spp.), has sometimes been very difficult because the cut surface of the explant taken from the mother plant may brown and the medium often darkens. When this process has started, it is difficult to save the culture. This process may be caused by the presence of tannins or other polyphenols present in the tissues that oxidize rapidly on the cut surface and inhibit morphogenetic activity. It is possible to overcome this problem using appropriate substances. In 1965, Jones (20) employed polyvinylpyrrolidone (PVP) to prevent polymerization of phenolic compounds. This compound had been employed successfully by Walkey (57) for apple shoot culture and by Christiansen and Fannesbech (9) for *Hamamelis*. However, browning of chestnut and walnut was not overcome with PVP.

In 1976, Jones (21) reported successful results with phloroglucinol on apple explants, and, more recently, improved results have been achieved with reduced glutathione (GSH), a tripeptide that has a low level of toxicity and can be added to the substrate. GSH was first used to prevent browning caused by growth regulators in olive cultures (41) and it has also been tested successfully on apple and chestnut.

The precise composition of culture media that varies with species and clones is not covered in this review. A satisfactory medium must permit normal morphogenesis and results must be consistent. All of this usually does not happen.

A serious problem is tissue vitrification (also known as glassiness or waterlogging), which often occurs in micropropagated fruit species. This term, which is seldom clearly defined, describes the morphological disorder of microplants that lose their capacity to propagate and/or present difficulties of acclimatization. The largest number of vitrification symptoms seem to be associated with high-salt MS medium, which is the most commonly used; however, some ions (such as K and Mg) do not seem to effect vitrification (37, 38, 39).

Many tissue culture laboratories have had to deal with vitrification because it results in great losses in material and disruption of the production cycle. Kevers et al. (24) proposed that vitrification results from a burst of ethylene production, controlled by the peroxidase–IAA–oxidase system. The increase in ethylene production would be determined by an initial stress. The final result of these processes is decreased cell wall lignification and cellulose synthesis—both of these factors would allow more water uptake, which results in hyperhydric malformations. Pasqualetto et al. (39) showed that the number of stomata were greater in vitrified than normal leaves, confirming that vitrification is a water-related problem.

However, from a practical point of view, vitrification can be cured by increasing the agar concentration or reducing cytokinin level, but the proliferation rate diminishes.

Pasqualetto et al. (38) proposed that vitrification of apple shoots could be overcome and proliferation rate maintained by mixing Gerlite with agar at a certain concentration. Commercial in vitro plant production will not be completely efficient until the phenomenon of vitrification is entirely understood and controlled.

A number of physical and chemical factors within the culture medium relate to growth and morphogenesis; the most easily isolated variable to take into account is pH.

Medium pH usually is adjusted around 5.5 before autoclaving. Recent studies on three different media (MS containing sodium hydrate-citrate buffer, MS, and a modification of MS called PM) (29) with pH values adjusted to 5.5 before autoclaving (120°C for 10 min) produced pH values of 5.5, 5.3, and 5.2, respectively. Such a change is not really negligible and reveals strong variations in the balance and level of chemical substances that affect the availability and effectiveness of growth regulators. These variations probably increase with changes in temperature and length of sterilization. This change is important because it means that the single operation of autoclaving can determine differences even within the same basal medium. In addition, pH values change continuously once the culture has started. Within a week, pH values may drop 1.5 units;

pH rises again afterwards with different velocities and may reach neutrality. These variations not only depend on the medium itself but, what is more serious, they are strongly influenced by the relationship that exists between the mass of the medium and the mass of the explant. During culture, the medium concentration varies continuously. There is a slight concentration effect due to water loss, but this phenomenon tends to be suppressed by opposing processes such as respiration and absorption, which lead to dry matter elimination from the medium.

There are many other problems that arise when in vitro techniques are brought into nursery application. A key problem is improved conditions for acclimatization from the in vitro to in vivo environment. These include environment (light, temperature, and humidity), chemical (pH and medium composition), and sanitary factors that are critical during the first days after transplantation.

NURSERY APPLICATIONS

In spite of many problems, in vitro propagation has gained a place in the nursery industry of countries with advanced technology (Europe and North America in particular). In Italy, micropropagation is becoming important for the fruit industry, especially for the production of clonal rootstocks that are difficult to propagate with traditional methods.

Much research has been conducted on this technology, and there is now sufficient expertise to permit us to obtain repeatable results even if the physiological mechanisms are not determined. The number of cultivated fruit trees that can be propagated in vitro is continuously expanding.

In addition to experimental results obtained in well-equipped research laboratories, tissue culture is a technique commercially employed for the propagation of a great quantity of material of several plant species including medicinal, ornamental, vegetable, fruit, and forest crops. The progress made in fruit crops in the last 5 to 6 years is remarkable and millions of fruit plants in Italy are produced annually in vitro.

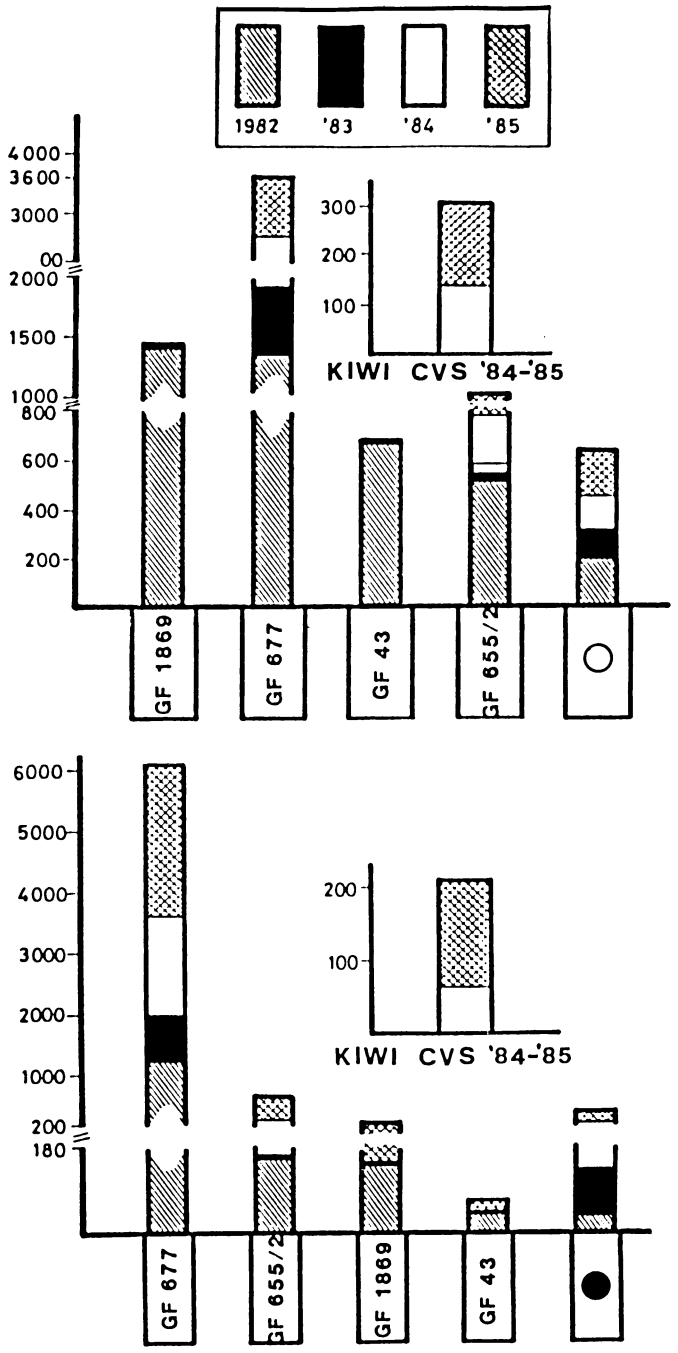

Fig. 1. Number ($\times 10^3$) of clonal rootstocks or cultivars produced in vitro in two commercial laboratories in Italy. (Top) Vitrocoop (1977); (bottom) Battistini (1980). For both laboratories the year of the first commercial production is indicated; from beginning until 1982 production is cumulative. (○) Other clonal rootstocks, self-rooted kiwi and peach. (●) Other clonal rootstocks, self-rooted plum and kiwi.

Species and numbers obtained through tissue culture

There were four laboratories in 1985 that produced in vitro fruit plants in the northern and central regions of Italy. One of these is organized to produce material free from the more common viruses. For the immediate future, the number of commercial laboratories will increase by three or four units, spread also in the southern part of the country.

Micropropagation is used mainly to produce clonal rootstocks of apple and peach (Figs. 1 and 2), the two species of most economical importance. In addition, their rootstocks have great commercial value.

For instance, the number of micropropagated dwarfing apple rootstocks that normally are difficult to propagate with the traditional techniques, such as M 27, M 26, M 9, together with MM 106 and MM 111, reached 3 million at the end of 1985. With peach, the largest proportion of this production is represented by the rootstock GF 677, a peach–almond hybrid that is Ca resistant, as well as by other clones (such as INRA GF 1869, INRA GF 43, and INRA GF 655/2) having characteristics that adapted them for use not only with peach but also plum and apricot. These clones have been produced in large amounts—more than 20 million plantlets.

This technique has permitted Italy, for the first time in many years, to supply the export market. Until 1979, Italy was one of the main buyers of clonal rootstocks on the European market, in particular from France, Holland, and the United Kingdom. Tissue culture has altered this situation dramatically.

Other clones propagated by tissue culture include 'Pixy' (a clonal rootstock for cherry) and 'Hayward' kiwifruit [*Actinidia deliciosa* (A. Chevalier) C.F. Liang et A.R. Ferguson]. Kiwifruit is practically the only scion cultivar propagated by this technique. It is possible to produce own-rooted apple, peach, plum, and cherry cultivars, and a small number has been produced in commercial laboratories. The limiting factor has been

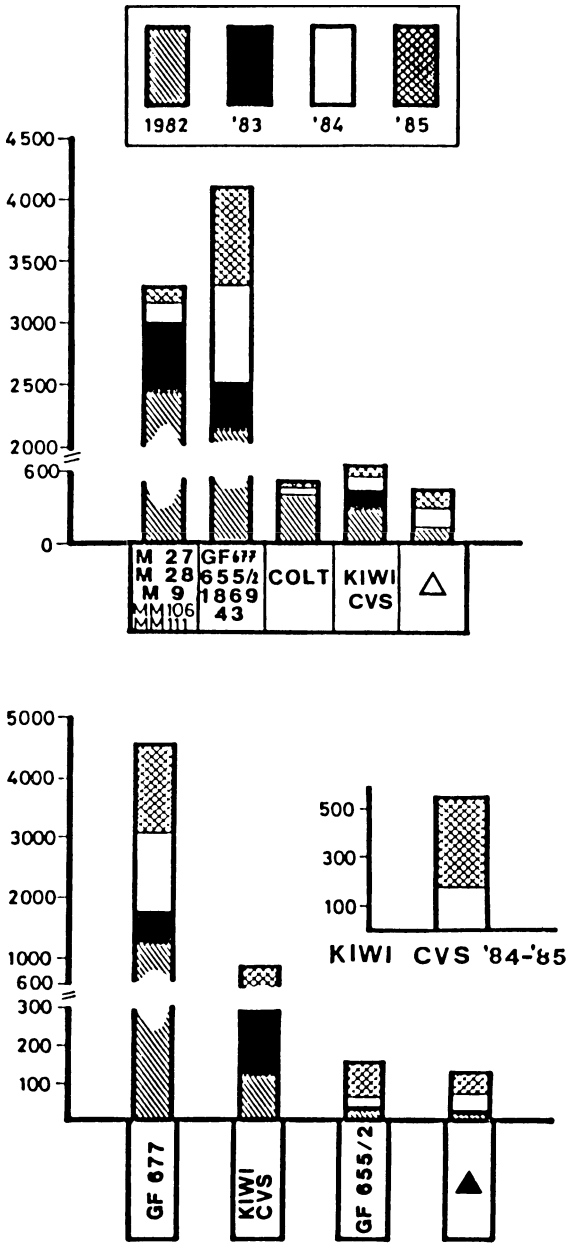

Fig. 2. Number ($\times 10^3$) of clonal rootstocks or cultivars produced in vitro in two commercial laboratories in Italy. (Top) Zanzi Vivai (1979); (bottom) Vitroplant (1980). For both laboratories the year of the first commercial production is indicated; from beginning until 1982 production is cumulative. (△) Other clonal rootstocks, self-rooted plum and kiwi. (▲) Self-rooted peach.

knowledge of their performance as own-rooted trees.

The results of preliminary studies on the agronomic behavior of own-rooted cultivars (32, 58) are contradictory in some cases. With peach ('Redhaven', 'Silver Logan', 'Fantasia'), for instance, earlier production and higher yields are obtained as compared to the same cultivars grafted onto a rootstock; while with apple ('Cox's Orange Pippin', 'James Grieve', 'Greensleeves', and 'Golden Delicious'), grafted trees had, in general, better performance.

Methods and media employed

In Italy, laboratories that produce tissue-cultured plants on a commercial scale employ the axillary bud method. Media formulations used by individual laboratories are proprietary and recipes are guarded jealously.

An aspect that still needs to be improved is the production of material that can enter a production cycle that corresponds to the natural rhythm of growth and market demand. This aspect requires a high level of organization. Limiting factors are the lack of control of phenomena such as vitrification, as well as problems related to acclimation.

In normal conditions with fruit plants, acclimation requires from 20 to 40 days. Sometimes, material enters into a quiescent stage after the first growth phase that usually lasts a few days. In the second phase, growth usually continues rapidly and can be increased if adequate cultural practices are provided. An interesting solution has been found to solve the problem of the optimum time to acclimatize plantlets (the best season is between March and September); so plantlets that are produced in winter are refrigerated until spring when they are acclimatized and transplanted. This technique has become normal practice (49).

CONCLUSIONS

In vitro production has resulted in about 20 to 25 million plants since 1985, and this number is increasing. The increased use of micropropagation has been due to economic pressures to reduce production costs (especially for rootstocks that are difficult to propagate economically by standard practices), to satisfy a demand for clonal rootstocks that is becoming more and more specific, and to produce a great quantity of material in a small space, starting from a few mother plants.

If the research underway on the variability and performance of self-rooted clones gives positive results, commercial laboratories will not be able to satisfy national demand without increasing facilities and the way will be open for the expansion of new facilities. There are great possibilities for micropropagation, but problems such as genetic stability and physiological defects such as vitrification remain to be solved by this young industry.

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