Achievements and Trends in the Use of Tissue Culture for the Mass Propagation of Fruit Plants and Germplasm Preservation at the Research Institute for Fruit Growing, Pitesti, Romania

Received for publication, December 15, 2009
Accepted, January 10, 2010

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Abstract

The use of tissue culture in the regeneration and commercial propagation of economically important plants is a comparative recent and radical development. Advances in biotechnology provided new methods for rapid production of high quality, disease-free and uniform planting material. Biotechnological tools like in vitro culture and micropropagation offer a valuable alternative in fruit trees propagation studies, virus control and management of genetic resources. At the Research Institute for Fruit Growing Pitesti the technique of in vitro micropropagation was employed since 1975. Its objectives were virus elimination from certain strawberry cultivars by meristem culture, rapid propagation under aseptic conditions and in vitro preservation of strawberry germplasm. Subsequently, the tissue culture research was extended to many other fruit species, such as blackberry, raspberry, currant, gooseberry, apple, pear, quince, plum, sweet cherry, and sour cherry. The applied research was focused on the improvement of in vitro technologies for fruit and ornamental species, oriented mainly towards optimization of the culture media and shortening the period for producing in vitro plants at low costs. Based on the results of tissue culture research and advances in application of in vitro techniques, a range of micropropagation technologies were established, which enabled high quality and efficient plant production at commercial scale. Remarkable achievements have been made also in the medium and long-term in vitro maintenance of small fruit germplasm under cold storage conditions. In this review we will summarize advances in the application of plant tissue culture to fruit tree species, and highlight the achievements made in the last 25 years at the RIFG Pitesti in meristem culture, micropropagation, and germplasm preservation.

Keywords: fruit species, tissue culture, micropropagation, in vitro preservation, cold storage

Introduction

The horticultural applications of tissue culture are numerous; however, three broad classes of application can be recognized: plant propagation (micropropagation), genetic manipulation (genetic transformation and mutagenesis), and product synthesis (enzymes, secondary metabolites including pharmaceuticals, dyes, and fragrances). In fruit species, the various in vitro techniques (called biotechnologies) have found numerous practical applications both for the clonal propagation and genetic improvement. As a biotechnology application, the micropropagation is the most recent method employed for the commercial plant propagation in horticulture and forestry. This technique developed more than a half-century ago and applied commercially since the ’70s, when several tissue culture laboratories aiming at the mass clonal propagation were established in some countries, became indispensable and is now widely used with an impressive number of plant species.

In Romania, strawberry was one of the first plant species introduced in the in vitro culture, aiming at the developing of rapid and efficient procedures for mass clonal
propagation. In less than a decade from the first attempts (1975) to micropropagate the strawberry at the RIFG Pitesti, a complex of commercial and research laboratories was established. The Second Symposium on Plant In Vitro Culture held at the RIFG Pitesti in 1983, marked the inauguration of these laboratories, at their beginning engaged solely in micropropagation of strawberry cultivars for assuring the stock of planting material for the whole country.

In the ’90, the researches were extended to many other fruit species, aiming mainly to the obtention of virus-free plants. Also, sustained efforts were made towards the development and verifying of biotechnology procedures for efficient propagation of the new created fruit varieties.

After 25 years from its establishment, the primary applications within the tissue culture laboratory are: (1) mass propagation of specific clones, often used for building up the stock needed for introduction of new cultivars; (2) production and maintenance of pathogen-free (indexed) plants, often as the initial step in a nuclear stock plant production scheme; (3) germplasm preservation; (4) year round production of plants.

The overall research work carried out within the tissue culture laboratory has been driven both by the scientific interest for developing and improving the biotechnologies for high quality and efficient propagation of fruit plants, and by the commercial demands.

In the present paper we review our own results and achievements from the last 25 years of tissue culture research in small fruit and fruit tree species.

**Applications in the field of planting material production**

1. **Meristem culture for virus elimination**

Viral diseases propagate easily in fruit plants and causes both yield decrease and poor quality of fruits. The interest for producing virus-free plants increased constantly based on the well known fact that most often is difficult to cure and restore the health of infected plants. The plants obtained from meristem cultures can be directly used, but most often they are used as mother plants for producing healthy planting material by the conventional vegetative propagation. In some fruit species (e.g., strawberry and raspberry), the method of *in vitro* meristem culture is generally employed for virus eradication in the biological material used for commercial propagation. Regeneration of virus-free plants is inversely proportional to the size of used explant. Frequently, this is associated with thermotherapy, but it is considered that the majority of viruses known at present can be eliminated from the fruit plants by meristem culture without the employment of thermotherapy, provided that the excised meristem tips are less then 1 mm in length. However, we found that the efficiency of virus elimination is always higher when meristem explants with a size of 0.2-0.5 mm are used.

In strawberry, the efficiency of regeneration process and *in vitro* multiplication of the healthy material, consequence of the improved culture media and methods of cultivation, are major advantages in successful eradication of viruses from fruit varieties grown commercially. The range of viruses with high incidence in strawberry plants, which are currently eradicated by plant regeneration from meristems, include the following: latent A strawberry virus, strawberry vein-yellowing, strawberry crinkle virus, strawberry mild yellow-edge virus, strawberry mottle virus. Within the tissue culture laboratory at the RIFG Pitesti, virus elimination from strawberry has already a long history, being initiated by COMAN & al. [1] with Redgauntlet, Gorella and Senga Sengana varieties, using explants of 0.1-1.5 mm excised from unrooted runner tips and cultured onto the medium recommended by BOXUS [2].

The observations have shown that the size of explants influences both their ability to grow and differentiate into shoots, and the frequency of the resulted healthy plants. Only 42-
70% of the meristem explants, depending on the strawberry variety, have developed into shoots, but they were all giving rise to healthy, virus-free plants.

In raspberry, one of the small fruit species to which the virus infections cause high yield losses, the viruses that can be eliminated by meristem culture include: raspberry leaf spot virus; raspberry ringspot virus; raspberry leaf mottle virus; raspberry mosaic and rubus yellow net.

After GHENA & al. [3], who published in 1978 the results of first attempts carried out in Romania for eliminating viruses from raspberry cultivars, COMAN & al. [4] reported the obtention of virus-free planting material in both strawberry and raspberry. Thus, they obtained plants free of the raspberry mosaic virus in several raspberry cultivars by using thermotherapy (for 30 days), followed by culture of meristems.

The results of subsequent investigations have shown that the success of meristem culture, as method for eliminating viruses from the raspberry cultivars, is conditioned by the use of optimized culture media and hormone combinations (ISAC [5,6]), as well as the significant influence of the moment of collecting explants and size of explants. It was also found that the genotype might be equally a very important factor influencing the regenerative response of in vitro cultivated meristems (ISAC [7,8]).

Between 1990-2000 the work of in vitro cultivation of meristem explants having various sizes for eliminating viruses from raspberry and blackberry cultivars led to the obtention of virus-free plants, especially when meristems of very small size were used (ISAC [9]). Thus, in vitro culture of meristems smaller than 0.1 mm allowed regeneration of 100% plants free of raspberry ringspot virus in raspberry cultivars Ljulin, The Latham, Autumn Bliss and Glen Prosen, and respectively in blackberry cultivars Silvan and Darrow. Moreover, the latter cultivar was also proved to be free of raspberry leaf spot virus. Results of subsequent researches revealed that a small size of the meristem explant is also an essential condition for eliminating the RLMV and RLSV from raspberry cultivars such as Newbourgh, Bulgarski Rubin, and Romy. However, it was found that in the case of infection with raspberry bushy dwarf virus (RBDV), the size of meristem explants did not influenced the elimination of virus from cultivars such as Lloyd George and Schoenemann. When meristem explants with the size of 0.1-0.3 mm were cultured in vitro, depending on the virus and raspberry cultivar, all regenerated plants were virus-free, or only part of them (over 40%). The meristem explants of higher size (0.3-0.5 mm) were proven to be unsuitable for virus elimination, irrespective of the virus and raspberry cultivar.

In woody fruit species, the first attempts for eliminating viruses from infected cultivars by the use of meristem culture were made in 1983, although the studies aiming at the in vitro multiplication were initiated since 1981 (ISAC [10,11,12]). The researches carried out contributed to a great extent to the establishment of best moment for collecting biological material and optimal in vitro culture conditions for meristems, allowing the obtaining of virus-free plants. The most favorable period for collecting meristem explants was found to be from November to February, when their physiological state allows the best regenerative response. Results obtained with a range of sweet and sour cherry genotypes, such as Van, Cerca, Meteor, Oblacinska, Nana, Colt, F12-1, IP-C1 (ISAC [13]), aiming at virus elimination, have shown that the degree of virus infection decreases in subsequent subcultures. Also, it was found that the culture media containing cytokinins in high concentrations allows virus elimination in the highest degree. Based on these findings, ISAC [14] emphasized that there is the possibility to eliminate viruses from the sweet and sour cherry cultivars (including rootstocks) during micropropagation, provided that adequate media are used, and this is achieved in a relatively high number of subcultures. ACLSV proved to be the easiest virus to eliminate by meristem culture in sweet and sour cherry.
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(100%), while PDV and PNRSV are more difficult to eliminate (81.1-94%) even when the meristem is as small as 0.1 mm. The technologies developed by us for eliminating PDV, PNRSV and ACLSV from sweet cherry and sour cherry plants involves a relatively high number of subcultures (4-5) on the Murashige-Skoog (1962) solidified medium supplemented with 4.4 µM benzyladenine (BA), 0.46 µM kinetin (Kin) and 0.49 µM indolebutyric acid (IBA). A similar technology was developed for eliminating PPV and PDV from the plum cultivars, by using Lee-Fossard (1977) solidified medium supplemented with 8.8 µM BA and 2.9 µM gibberellic acid (GA3), (ISAC & al. [15]).

At present, within the Tissue Culture Laboratory of RIFG, the elimination of viruses is completely integrated with clonal production of planting material in the most of small fruit and fruit tree species.

2. Clonal propagation of fruit cultivars; the application of the micropropagation biotechnologies on large scale

Micropropagation is a simple concept and now is considered a routine technology. The steps in micropropagation method are: (a) Initiation of culture - from an explant like shoot tip on a suitable nutrient medium; (b) multiple shoots formation from the cultured explant; (c) rooting of in vitro developed shoots and, (d) transplantation to the field following acclimatization. The scientific literature is strewn with papers reporting yet another protocol for a particular species or cultivar.

The production of high quality and healthy planting material created new opportunities in the global economy for producers, farmers, and nursery owners. For instance, the mass production of strawberry plants micropropagated in vitro, introduced for the first time in Belgium, offered an improved way as alternative to the slow and very strict scheme of plant certification. Therefore, this method was adopted by the most of European fruit growers within the next five years.

In Romania, the remarkable achievements in strawberry mass propagation, and the establishment of Tissue Culture Laboratory, led to new and systematic approaches on in vitro culture of explants in numerous small fruits and fruit tree species with economic importance. Among them, the apple was particularly investigated, for either obtaining cultivars on their own roots as planting material, or propagating the vegetative rootstocks in order to produce the initial material needed for layering propagation.

Based on the results obtained from the experiments aiming at the in vitro rooting of D1R57T120 apple hybrid, selected at the RIFG for its resistance to both apple scab and mildew, NECULAE & al [16] reported that the type and concentration of auxin present in the culture medium have a strong influence on the ability to form roots, and also on their growing in length. Subsequently, this finding was confirmed by the results of investigations carried out with some other apple cultivars cultivated in vitro, such as Pionier, Generos and Lodyspur, which revealed differences in both the overall response to aseptic culture, and rooting ability of the microshoots (NECULAE & al [17]). As NAJIM & al [18] reported previously, the highest micropropagation rate in the MM106 apple rootstock can be obtained on the media lacking auxin, provided that BA at a concentration of 8.8 µM is present. The findings of the research team from RIFG Pitești led to a significant improvement of the media used for clonal propagation of both apple scions and rootstocks.

The researches aiming at the improvement of protocols employed for micropropagation of woody fruit species were intensified after 1991. NECULAE [19], reported that ratio 5.4 µM naftylacetic acid (NAA): 5.3 µM BA as being the most efficient for the in vitro propagation of MM106 apple rootstock. These findings were confirmed soon by ISAC [20,21], who emphasized also the strong influence of the number of subcultures,
reporting that after the fourth subculture of M9 apple rootstock a multiplication rate as high as 43 microshoots/explant was obtained. The results of the studies carried out by NECULAE & al [22] with the MM.106, M.26, and M.9 apple rootstocks, which showed that rooting percentages higher than 75% can be obtained on culture media supplemented with 2.46 µM IBA, were of great importance for improving the efficiency of in vitro clonal propagation of all the apple rootstocks used in Romania.

The studies aiming at establishing the influence of light during the stage of in vitro micropropagation of the MM106 apple rootstock have shown that the light period can be reduced from 16 hours to 10-12 hours without any negative effect on the multiplication rate and quality of obtained plants, provided that the light intensity is maintained at 3.000-4.000 lux (NECULAE & al [24,25]). Based on the results obtained with the same apple rootstock, (TEODORESCU & al [26]) reported that maintaining of micropropagated shoots in darkness for 9-14 days after their transfer on the rooting medium, prior to applying a light period of 12-14 hours, does not hamper the rhizo genesis process. By reducing the day light period to 10-12 hours during the stage of micropropagation, an up to 20% saving to the cost of energy became possible.

The improvement of the protocols used for the mass propagation of these apple rootstocks was further enabled by the results of studies carried out by TEODORESCU & al [23], showing the influence of the abiotic factors such as pH of the substrate and air humidity during the preacclimatization and acclimatization on the plant survival ex vitro.

Similar investigations carried out with some pear rootstocks (Alamâi, Harbuzești, Cămârie, Românoască, PC56) cultivated in vitro resulted in establishing the most favorable nutrient media for their multiplication and rooting (NECULAE & al [27]). Significant differences were found in their response to in vitro culture, PC56 showing the best ability to micropropagate, especially on medium containing BA in concentration of 8.8 µM and NAA in concentration of 2.69 µM, respectively.

In plum, the response of various genotypes to in vitro propagation was studied by ISAC [28], who established the best auxin: cytokinin ratio for each of them. A great variation was found in their genetic potential to micropropagate, ranging in average from 3 microshoots/explant in cultivar Anna Spath to 14 microshoots/explant in cultivar Centenar.

As genetic variation can occasionally occur in interspecific or even intraspecific hybrids having a high ploidy level, in parallel with the establishment of the most appropriate culture medium for the plum hybrid Tuleu Timpuriu x 5/23B, selected for some characters not found at its parents, NECULAE & al [29] confirmed the absence of any variation in the chromosome number.

An important contribution to the in vitro propagation of sweet cherry and sour cherry was brought by ISAC [30,31], who established the best basal media (Lee-Fossard, Lepoirve, Anderson, Walkey) for many scion and rootstock cultivars. Thus, sweet cherry cultivars such as Bing, Sam, Cerna and Stella, and sour cherry cultivars such as Chatenmorelle, Nana and Meteor, were easily multiplicated on media containing 4.4 µM BA and 0.87 µM GA, and then rooted on media supplemented with indolylbutiric acid (ISAC [32]).

An important task for the researchers working within the Tissue Culture Laboratory at the RIFG Pitesti was to develop the most appropriate protocols for large scale micropropagation of cultivars introduced from abroad or created in Romania, allowing their rapid introduction into commercial growing. In this respect, after 10 years from the first reports on micropropagation of some promising cultivars of strawberry and raspberry (COMAN & al [33,34]), the research work aiming at studying the response of some new cultivars to micropropagation has been resumed (TEODORESCU & al [35], and then to optimize the culture media and photoperiod.
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Based on the results of these studies, the protocols used for micropropagation of strawberry and blackberry cultivars could be optimized both in terms of efficiency of in vitro clonal propagation, and cost per micropropagated plant. In this respect, the amounts of cytokinin and auxin used in the stage of multiplication have been reduced and the sucrose was replaced as carbon source with dextrose, the last being produced in Romania and purchased at prices 5-10 times lower (NECULAE & al [36,37,38]).

The research towards the reduction of cost per micropropagated plant had the elimination of the in vitro rooting stage as an important objective. The results obtained by TEODORESCU & al [39] from experiments carried out with apple rootstocks, strawberry and blackberry, revealed that many cultivars have a good potential for ex vitro rooting of the micropropagated shoots, especially in blackberry in strawberry. More recent researches resulted in establishing improved technologies for in vitro clonal propagation of some thornless blackberry cultivars introduced in Romania in the last decade. These are based on the optimization of factors such as photoperiod and light intensity, as well as on the replacement of in vitro rooting stage with the rooting of microshoots under septic conditions into perlite substrate. Although cultivars such as Hull, Black Satin and Kotata were proven to have a good response to the in vitro culture, significant differences were found in their behavior during the different stages of clonal propagation.

In the recent time, various aspects of raspberry tissue culture have been systematically studied to develop an efficient micropropagation regime and improve culture methodology. The studies done by ISAC [40,41] revealed that the Murashige and Skoog medium assured in vitro growing conditions superior to those offered by the Anderson medium for all the raspberry varieties cultured in vitro in our laboratory. On the MS media with 0.49 µM IBA, 13.2 µM BA and ascorbic acid, multiplication rate is generally much higher. Although it is ascertained that high concentration of cytokinin may result in extreme, undesirable bushiness, 13.2 µM BA had positive effects in most raspberry varieties micropropagated in our laboratory.

Works on in vitro rooting of raspberry microshoots revealed that the Murashige and Skoog medium containing half concentration of minerals and supplemented with 9.8 µM IBA and PG, is essential for ultimate expression of rhyzogenesis potential. The degree of in vitro rooted shoots ranged from 69.9% (‘Willamette’) to as much as 100% (‘Heritage’ and ‘Bulgarsky Rubin’), (ISAC [42, 43]). The results obtained over many years of study clearly reveal strong influence of genotype on the rooting ability. The experimental results regarding ex vitro rooting of the raspberry microshoots indicated that the percentage of plants rooted directly into the perlite substrate in the greenhouse was similar to that with in vitro rooting for varieties Bulgarski Rubin, Malling Exploit, Cayuga, Citria and Ruvi (ISAC [44], ISAC& al [45]).

With some small fruit species, such as currants (NECULAE [46]) and gooseberry (COMAN & al [47]), the research results were not good enough to allow the establishment of reliable and efficient technologies for their micropropagation. Aronia arbutifolia, a recently introduced small fruit species in Romania, was proven to be less recalcitrant to the in vitro culture, allowing the obtention of a good micropropagation rate (NECULAE [48]).

During the 25 years of research activity, the Tissue Culture Laboratory within the RIFG Pitești, an impressive technological capital has been accumulated, including biotechnologies for the in vitro micropropagation of strawberry, raspberry, blackberry, vegetative apple rootstocks, etc. The application of the new or improved biotechnologies allowed both increasing the number of micropropagated genotypes and the production of plants.
In strawberry, for instance, whose micropropagation is included in the new official scheme of producing healthy and certified planting material, an average of 8,000 plants of superior quality (Prebase) were obtained yearly in the period 1991-2009 for 8-10 cultivars, which were used as stock mother plants.

The average number of small fruit and fruit tree species propagated by tissue culture in the period 1992-2000 was double of that from the period 1980-1991. Also, there was a significant increase in the number of cultivars we have propagated clonally in vitro on large scale. In accordance with the increase of the number of species and cultivars, the production of in vitro propagated plants increased as well, especially as a consequence of the export demands for strawberry and blackberry.

By in vitro propagation, a total of 721,800 plants were produced in the period 1991-2000, with a maximum in 1995, when over 150,000 plants were produced. From the total amount of planting material produced by clonal micropropagation within the Tissue Culture Laboratory, 68.4 % was exported to Great Britain and France. Thus, 411,360 strawberry and blackberry plants were exported between 1994 and 2000.

Although the production of in vitro micropropagated plants decreased at about a half in the period 2001-2009, and a proportionally decrease of the export, over 421,000 vitroplants were produced, out of which about 236,000 were exported. An improved biotechnology protocol was developed in the above mentioned period for raspberry, which allowed the in vitro clonal propagation of about 9,100 plants from ten different cultivars.

3. Germplasm preservation

In vitro maintenance of germplasm under cold storage conditions was initiated at the Fruit Research Institute with in vitro storage of strawberry. Between 1978-1981 the whole strawberry collection including 125 cultivars was introduced in the in vitro culture on four nutrient media, in order to be preserved at temperatures of +2 or +4ºC. The results obtained indicated a different response of various cultivars depending on the nutrient medium, of practical importance being the fact that the strawberry germplasm can be preserved in vitro entirely for at least 10-12 months on media with rich content in mineral salts (COMAN [49]).

In the recent years in vitro maintenance of small fruit germplasm under cold storage conditions was done according to the principles of an in vitro active gene bank, all material flowing through a cyclical process of multiplication. From several reasons, including the financial one, the in vitro germplasm collection was strictly limited in recent years to the needs of commercial propagation, preservation of some rare genotypes, and also preservation of the genotypes currently used as indicators for virus infection in small fruits.

Long term storage of strawberry germplasm at low temperature is currently achieved on Lee & Fossard basic medium lacking any growth regulators. For raspberry and blackberry, the in vitro maintenance of germplasm under cold storage conditions is currently achieved on Murashige & Skoog medium, supplemented with small quantities of IBA (in raspberry), NAA (in blackberry), BA and GA3. At 4ºC, under sub-optimal conditions for in vitro growing, the time interval for subculturing event was established at about 3-4 months for raspberry, 4-6 months for blackberry, and 8 months to one year for strawberry, respectively. The in vitro storage of the small fruit germplasm could be achieved without any difficulties, the loss of material being only accidental. Moreover, there is no evidence for genetic instability among the plants obtained from the microshoots preserved in vitro under cold storage conditions. So far, the number of in vitro preserved Fragaria and Rubus genotypes was essentially limited to the needs for commercial micropropagation. A total of 124 genotypes are maintained under cold storage at 4ºC as in vitro microshoots, including 43 cultivars and clones of Fragaria x ananassa, 5 clones of F. vesca, 3 clones of F. virginiana, one cultivar of F. moschata, one
cultivar of *F. x vesca*na, *6 Fragaria* interspecific hybrids, 21 cultivars and 11 clones of *Rubus idaeus*, 23 cultivars of *R. procerus*, one clone of *R. occidentalis*, and one clone of *R. phoenicolasius* (COMAN & al [50]). At present, the biological material can be maintained *in vitro* for about one year without transfer onto fresh medium, under conditions of slightly reduced light and temperature. By decreasing the temperature, each stage of development can be stoped, and the plants can be preserved without affecting their capacity of subsequent development. From the already long time experience of the Tissue Culture Laboratory at RIFG Pitesti, this type of *in vitro* preservation can be extended to periods of several years.

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