PROTOCOALE VALIDATE PENTRU MICROPROPAGAREA UNOR SPECII POMICOLE ÎN VEDEREA PRODUCERII STOCURILOR DE PLANTE PREBAZĂ

VALIDATED PROTOCOLS FOR MICROPROPAGATION OF SOME FRUIT SPECIES TO PRODUCE PRE-BASIC PLANT MATERIAL

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Abstract

Obtaining virus free fruit planting material, conservation and utilization for large scale production of certified fruit trees, was and is still an objective demand. It knows already that significant cultural performances achieved by fruit trees, are direct related to the biological value of planting material used. Often it is necessary to introduce rapidly in culture new varieties and, this it is possible through tissue culture. Tissue culture is commonly called "cloning" or "micropropagation". In horticulture, the micropropagation is the most recent method used for the commercial plant propagation as a biotechnology application. At Research Institute for Fruit Growing Pitesti Argeş strawberry was one of the first plant species introduced in the in vitro culture. In the `90, extensive research in many other fruit species, have to develop rapid and efficient procedures for mass clonal propagation. Also, sustained efforts are towards the development and verifying of biotechnology procedures for efficient propagation of the new created fruit varieties. Propagation systems approved by actual legislation regarding production of fruit tree planting material include also micropropagation as propagation technique. Data presented in this paper describe validated protocols for micropropagation of some varieties of strawberry and raspberry. These protocols tested had favorable results for more than five years and can thus be considered validated. This paper presents the data regarding aseptic cultures establishment, culture media, hormone combinations, culture conditions and specific technical parameters for fruit species and the role and importance of tissue culture in certification schemes.

Keywords: tissue culture, protocol, micropropagation, strawberry, raspberry
Cuvinte cheie: culturi de tesuturi, protocol, micropropagare, căpşun, zmeur

1. Introduction

Development of *in vitro* tissue culture techniques has demonstrated its application in rapid clonal propagation, regeneration and multiplication of selected genotypes. The tissue culture methods of plant propagation, known as 'micropropagation' utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium. The regeneration of plantlets in cultured tissue was described by Murashige in 1974. Micropropagation via direct shoot regeneration from meristems is well suited to obtain genetically homogenous planting material, identical with mother genotype. In fruit species, this technique assume high practical importance because is enabled not only rapid and effective plant propagation, but also elimination of pathogenes and this way production of healthy planting material. At Research Institute for Fruit Growing Pitesti Argeş strawberry was one of the first plant species introduced in the *in vitro* culture, aiming the development of rapid and efficient procedures for mass clonal propagation (Isac et al., 2010). The researches were extended to many other fruit species, aiming mainly to the obtention of virus-free plants for raspberry for example (Isac, 1997, 2004). Also, sustained efforts were made towards the development and verifying of biotechnology procedures for efficient propagation of the new created fruit varieties. Propagation systems approved by actual legislation regarding production of fruit tree planting material include also micropropagation technique. Fossard (1987) gave a detailed account of stages of micropropagation. The present paper is focused on description of efficient *in vitro* regeneration and propagation protocols for strawberry and raspberry. The paper presents the entire process from plant material to meristem establishment, past multiplication and rooting to acclimatization of plants. These protocols have been used for propagation of various varieties of these species, more than 10 years with good results and can thus be considered validated.

2. Material and Methods

2.1 Materials

For all protocols it is necessary to have some common materials and facilities: calcium hypochlorite $(CaOCl)_2$, sterile distilled water, 94° ethanol, orbital shaker, Erlenmeyer flasks, glass test tubes, 500 ml jars like culture vessels, pipettes, forceps, scalpels, stainless steel surgical blades, culture media, plant growth regulators, agar, specific biological material (buds, shoot tips, regenerated apical or axillary shoots), fully controlled environment tissue culture growth room, laboratories, greenhouse for acclimatization.

2.2 Methods

The protocols would require: a) initiation of culture or explant establishment - from either dormant or active buds, or shoot tips on a suitable nutrient medium, b) multiple shoots formation from the cultured explant, c) rooting either *in vitro* or *ex vitro* of developed shoots and, d) acclimatization or hardening off. All sterile manipulations were carried out in a laminar flow cabinet. Culture media are solidified by adding 7 g/l agar. Plant tissue culture media are sterilized by autoclaving at 121°C and 1.5 bar (150kPa). Sterilization takes 20 min. *In vitro* cultures were maintained in the growth chamber at 22-24°C, and photoperiod of 16/8 h of light/darkness. Light intensity provided by white fluorescent lamps ranged between 32-40 µmol m^2s^1. Initiation phase requires special attention on the part of the technician. It is highly recommended that a highly-trained and experienced person performs the task. To avoid contaminantion it is best to start cultures singly in test tubes.

3. Results and discussions

STRAWBERRY MICROPROPAGATION PROTOCOL

This protocol for *in vitro* multiplication of strawberry includes all the four stages.

Used cultivars

Premial, Real, Magic, Redgauntlet, Benton, Floral, Elsanta, Marmolada, Idea, Everest, Record, Senga Sengana

Culture media

The culture media for strawberry micropropagation is currently completely under control. The medium for shoot induction and multiplication is MS nutritive medium (Murashige and Skoog, 1962) containing kinetin - N^6-furfuryl adenine (K) and indole-3-acetic acid (IAA) for initiation of culture, and N6 - benzyladeniene (BAP) in combination with IAA for buds proliferation. For *in vitro* rooting of plants medium is composed of ½ MS macroelements, LF (Lee and de Fossard, 1977) microelements, MS vitamins with gibberellic acid (GA_3) and 3 – indolylbutiric acid (IBA) (Coman and Neculae, 1981; Teodorescu and Neculae, 1994; Neculae, 1996), (Table 1).

Initiation of culture – explant excision and sterilization

The original strawberry plant material is provided by strawberry breeder responsible with genetic conformity of a cultivar. Quality micropropagation is related to the availability of homogenous plant material at the initial step of the process. Runner tips about 2 cm were collected in early June from a field strawberry plants before runners develop new leaves and roots. It is easier to excise from young runner tips than from well developed plants as there are hairs in the meristem zone. Runner tips from each cultivar must be keeping in a separate bag and labeled.

Procedure for surface sterilization involves the following steps:

1. Washing samples under running tap water;
2. Dipping samples for 4 min in an 94% ethanol;
3. Dipping samples for 8 min in a calcium hypochlorite solution 6%;
4. Washing with sterile distilled water.

Meristems are further excised one by one using fine forceps and pieces of razor blade.The explant size should be < 0.3 mm so as to ensure sanitary status of the plant production line (Boxus, 1974). Apical tips (larger than 0.3 mm) or axillary buds can also be cultured by the same methods but will not exclude foliage nematodes and so must be taken from nuclear stock plants tested for foliage nematodes (OEPP/EPPO, 1998).They are asepticaly placed in test tubes containing 2 ml culture medium. About a month later the excised explant tips develop into a rosette of leaflets. They need to be transferred to the multiplication medium and now explants need to be carefully cleaned by removing all necrotic parts, callus, and roots. Independently of the variety more than 80 % of well - excised meristems develop into shoots (Redgauntlet 95%, Benton 96%, Premial 93%, Real 91%, Magic 86%, etc.).

Shoots proliferation / multiplication

The new rosette of leaflets it is transferred to culture vessel containing 20 ml multiplication medium, sealed with caps. Within the first 3 to 4 weeks, 2 to 3 new buds appear one by one at the base of the oldest leaves. These young axillary buds grow very quickly and, in turn, produce secondary axillary buds that cover the entire surface of a culture vessel. The initial rosette becomes a cluster consisting of 12 to 37 small buds. Each bud shows several short petioles with a small unifoliate leaves at the edges. At this stage, to progress in the clonal propagation buds can be aseptically separated and transferred onto a fresh multiplication medium. Every 4-6 weeks, each isolated bud will produce averagely 20 to 35 new axillary buds. Clusters are divided roughly into 4 to 5 small tufts (clusters) of buds that are transferred onto fresh proliferation medium. About 4 weeks later large clusters of new buds are formed and can be divided. Up to 10 multiplication steps can be achieved, but this figure should not be exceeded (OEPP/EPPO, 1998). Monitoring the transfers prevents the vitroculturist from overlaying the tenth subculture and from the risks of multiplying stipular buds. A continuous high proliferation rate depends on the transfers that must be done after 3 - 4 weeks of *in vitro* culture. If it is not possible to transfer the plants either due to lack of media or manpower unavailaibility, culture vessels must be stored at +2°C. At this stage, it is possible to create a stock of plantlets that could then be used for the initiation of new propagation lines.

Rooting

Rooting phase is an important step in micropropagation proces. Plantlets are separate from clusters and transferred onto a BAP free medium containing indole butyric acid to favour rooting (Table 1). Roots will appear 10 -15 days later in parallel with first true leaves. Depending on variety the rooting rate is between 72 - 96 %. Within 5 – 8 weeks, well rooted plantlets with 2 to 4 cm height leaves and 1,5 to 3 cm long roots are ready for acclimatization.

Acclimatization and adaptation rate

Strawberries are one of the species with a great capacity to support stress of plantlets transfer to the septic life conditions. Acclimatization can be achieved with a perlite substrate and covered with a plastic sheet maintaining a critical 100% humidity in the atmosphere. After 4- 5 weeks, plants developed new leaves and the plastic sheet can progressively be lifted up. The average percentage of acclimatized plants is ranging between 80 and 95 %.

In vitro culture parameters of some strawberry cultivars are presented in Figure 2. Such plants are considered as *in vitro* propagation stock.

RASPBERRY MICROPROPAGATION PROTOCOL

This protocol for *in vitro* multiplication of raspberry includes all the four stages.

Used cultivars

'Ruvi', 'Citria' 'Gradina', 'Heritage', Cayuga', 'The Latham', 'Malling Promise', `Malling Exploit', 'Willamette', 'Gustar', 'Vely', 'Autumn Bliss' `Opal`.

Culture media

Within the range of basic culture media recommended for raspberry micropropagation, MS nutritive medium (Murashige and Skoog, 1962) allowed the highest rates of explant growth, shoot formation and rooting in the varieties we cultured. The results obtained from several comparative experiments have shown that it is not the basic culture medium, but the added growth regulators that are essential for inducing regeneration in raspberry varieties. 0.5 mg l gibberellic acid, 0.1 mg/l benzyladenine, 10.0 mg l ascorbic acid on MS basal salts and vitamins are suitable concentrations for explant growth. Depending on the variety, a concentration of either 1.0, 2.0 or 3.0 mg l^{-1} N6-benzyladenine (BA), with a constant rate of 0.1 mg l^{-1} 3-indolyl butyric acid (IBA) is conventionally used in laboratory work on raspberry multiplication. L-ascorbic acid (25.0 or 50.0 mg l^{-1}) is always added to the culture medium as an antioxidant, which minimises yellowing. The MS medium containing half concentration of minerals and supplemented with 2.0 mg l^{-1} IBA and 162 mg l^{-1} phloroglucinol (PG) is the best for inducing high rooting rate.

Initiation of culture – explant excision and sterilization

Cultivars may be selected as candidate material on the basis of trueness to type, vigor, pomological quality and absence of pest symptoms. Candidate material found free of viruses can be maintained and multiplied in vitro ((OEPP/EPPO, 1998). As biological material is used primocane shoots collected from mature plants grown under quarantine in an isolated insectproof gauzehause. The shoots are cut in small segments with 1 bud and then put in a separate plastic bag for each cultivar.

The recommendations for explant cleaning are:

1. put segments of shoots under tap water for 30 minutes for washing;
2. dipping samples for 4 min in an 94% ethanol;
3. dipping samples for 8 min in a calcium hypochlorite solution;

4. wash in sterile distilled water for 3x5 min.

Under binocular dissect the bud by removing away thin slices from the base up to the meristematic dome. The bud explants selected should have 1-2 leaf primordia. With a knife cut this tissue and place it in the test tube. *In vitro* cultivation of explants is governed by chemical composition of the used nutritive media, type and ratio of compounds and in the initiation phase also by the season when the biological material is collected. The average regeneration percentage for all raspberry cultivars is 75.2 % and is done in about 30 days. The rate of explants developing into viable shoots is always higher than 50% in all raspberry varieties when buds are collected between May and July, the best response being observed in 'Willamette', 'Heritage', 'Malling Exploit', 'Cayuga', 'Ruvi, 'Opal', and 'Citria'. However, in a vast majority of cultivars, the statistics confirms that the period from August to September is the most suitable for collecting buds that contain meristem with highest shoot differentiation capacity (Isac, 1997, 2004).

Shoots proliferation / multiplication

Transfer explants grown on initial nutrient medium to a nutritive media with 3.0 mg l BA and 0.1 mg l IBA to stimulate differentiation of axillary buds. The raspberry micropropagation takes place over 25-30 days with the MR of 15 to 41.9 plantlets / explant, depending on the variety. It is relevant that in laboratory routine micropropagation work more than two thirds of raspberry varieties have had MR higher than 6 reaching a maximum of 41.9 shoots per initial explant in 'Ruvi'. The tendency for high multiplication rate when using this *in vitro* culture medium was statistically confirmed in many varieties including 'The Latham', `Cayuga`, 'Opal' and `Gustar` in which the average MR values regularly exceeded 20 shoots per initial explant (Isac and Popescu, 2009).

Rooting

The results obtained over many years of the study clearly reveal strong influence of genotype specificities on the rooting ability. It is therefore relevant to emphasise that varieties such as 'Opal', 'Ruvi`, and 'Cayuga` show higher rhizogenesis potential on an *in vitro* culture medium supplemented with 162 mg l PG, irrespective of the IBA concentration. However, in the absence of IBA, or its presence at low concentrations (0.5–1.0 mg/l), PG does not affect the rooting ability of raspberry shoots. The rooting rate in *in vitro* micropropagated raspberry shoots is generally high (95%) in most of the varieties cultured. Raspberry varieties 'Opal, 'Citria', 'Gradina', 'Ruvi', 'Gustar, 'and 'Vely' had steady rooting performance, rooting rate being predominantly 100%. The quality of *in vitro* rooting was very good in most raspberries cultured in the laboratory, both in terms of number, length, and vigour of the developed roots. Random inspection of rooted shoots showed great inter- and intra-varieties variations in the number of roots. 'Opal' and 'Ruvi` were among the varieties that displayed the best i*n vitro* rooting ability (with an average number of at least 4.6 roots per shoot). Similarly, root length also varied considerably among most of the varieties. Thus, most commonly, the average length of roots formed by micropropagated shoots of 'Autumn Bliss' 'Opal` was about 6 mm, whereas that of roots formed by shoots of 'Ruvi', 'Gustar, 'Opal` and 'Willamette' was more than 20 mm.

4. Conclusions

The protocol for strawberry micropropagation uses a low concentration of hormone (only 0.4 mg/l BAP for example) only 5 propagation steps and composition of culture media prevent the formation of callus. In this situation, we can conclude that this *in vitro* multiplication can be satisfactory to produce certified material.

Most raspberries are difficult to micropropagate. Although the Anderson medium is extensively used for large-scale propagation of raspberry varieties, the largest number of plants formed per initial explant was obtained when these were cultivated on MS medium. Considering the average number of shoots formed per initial explant higher than 15 as being a good rate of multiplication, the protocol we are currently using ought to be considered as effective for *in vitro* clonal propagation of raspberry varieties.

Acknowledgements

This paper was supported by MADR, Sectorial Plan ADER 2020, Project ADER 2.2.7., Contract 227/2011.

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Tables and Figures

Table 1. Composition of culture media

Compound	Strawberry			Raspberry		
	Meristem	Multiplication	Rooting	Meristem	Multiplication	Rooting
Macroelements	LF	LF	MS 1/2	MS	MS	MS 1/2
Microelements	LF	LF	LF 1/2	MS	MS	MS 1/2
Vitamins	LF	LF	MS	MS	MS	MS
NaFeEDTA	32 mg/l	32 mg/l	32 mg/l	32 mg/l	32 mg/l	32 mg/l
Gibberelin (GA_3)	-	-	0.1 mg/l	0.5 mg/l	-	-
Kinetin (K)	1 mg/l	-	-	-	-	-
N6-benzyladeniene (BAP)	-	0.4 mg/l	-	0.1 mg/l	1 mg/l 2 mg/l 3 mg/l	-
Indole-3-acetic acid (IAA)	0.27 mg/l	0.27 mg/l	-			
3 – indolylbutiric acid (IBA)	-	-		-	0.1 mg/l	1 mg/l
Glucose	40 g/l	40 g/l	40 g/l	30 g/l	30 g/l	30 g/l
Ascorbic acid				10 mg/l	25 mg/l 50 mg/l	-
Phluoroglucinol (PG)	-	-	-	-	-	162 mg/l

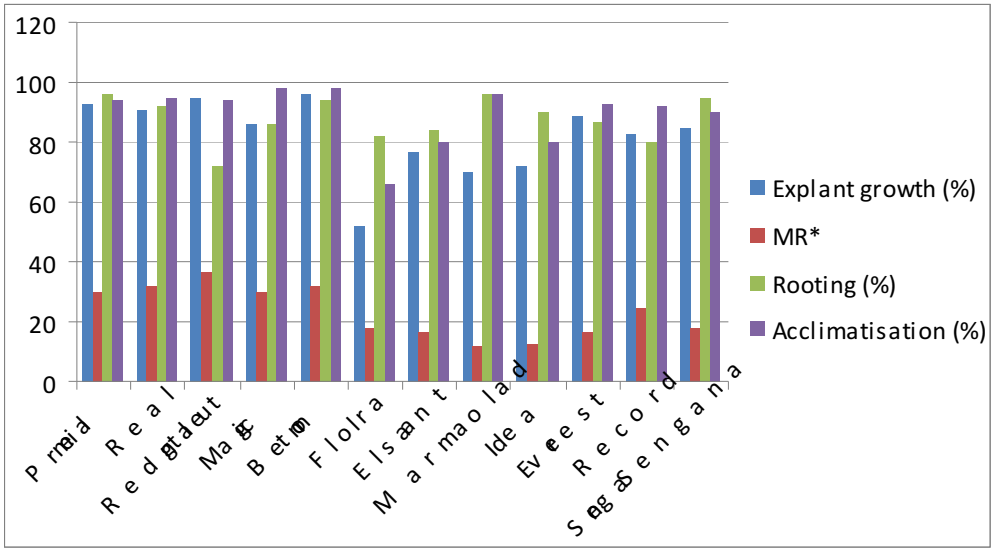

Fig.1 - Micropropagation parameters of some strawberry cultivars