Methods applied in the production system of *virus free* planting material of apple in the Republic of Moldova

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ABSTRACT

The technology of in vitro micropropagation of the apple rootstock MM 106 was developed. From 80 to 100% rooting was obtained in variants with indole-3-butyric acid (IBA) 1-2 mg/l. A diagnostic polyclonal antiserum to the apple stem pitting virus (ASPV) was obtained. The results of the virus elimination of apple cultivars 'Coreor', 'Coredova', 'Coretard', 'Izvoras' genetically resistant to scab *Venturia inaequalis* Cooke Wint using methods of thermotherapy are described. The virus free clone's category Prebase were planted in the depozitarium of the institute in order to increase and preserve the virus-free genefond of pome fruit crops.

Keywords: thermotherapy, micropropagation, antiserum, testing, clonal rootstock

INTRODUCTION

The production of apples, like other fruits, plays an important role in the economy of the Republic of Moldova. A significant reserve for increasing production and improving the quality of fruits is the effective protection of plantations from pests and diseases, among which virus and virus-like diseases have a special role. Direct crop losses from them are estimated at 20-25%. At the same time, the quality of products decreases (Calaşean *et. al.*, 2012). The general adaptability of the plant to unfavorable environmental factors decreases, especially to a moisture deficit in the soil and air, to sharp temperature fluctuations in the autumn-winter period.

Viruses negatively affect the survival rate of buds in the nursery and the yield of first-class seedlings, the quantity, and quality of cuttings of clonal rootstocks in the mother plant, and their ability to root. Some viruses cause abnormal development of pollen, its partial or complete sterility. This, in turn, leads to a constant decrease in fruit set in infected plants, which is especially acute in years with unfavorable weather conditions during the flowering period. It has been established that many causative agents of virus and virus-like diseases cause premature death of fruit plants. Often, affected trees develop a syndrome of incompatibility between the scion and rootstock, which leads to low productivity or complete death (Calaşean *et. al.*, 2012).

Virus diseases of fruit crops, unlike most fungal ones, are chronic and systemic. The use of infected trees as mother trees leads to the production of planting material from the very beginning, infected with viruses. (Verderevskaya and Kegler, 1980). At the same time, the disease remains "hereditary", and such seedlings will never be able to realize all the advantages inherent in this cultivar and rootstock.

It is known that chemical remedies used against fungal and bacterial diseases are not effective against viral infections. It is also known that viruses do not have their metabolism

and for reproduction, they use the same mechanisms and processes that are involved in the host plant. Therefore, all active substances capable of suppressing the synthesis of viruses in infected plants, at the same time, reduce productivity. Modern methods of dealing with them are based on the use of methods of healing and propagation free from overt and covert infection by viruses. By the method of micropropagation in vitro, cultivars and rootstocks of pome breeds rapidly multiply in the world (Chernets *et. al.*, 2003). Thermotherapy has been successfully applied against viruses from fruit crops and grapes.

Thus, diagnostic methods are of great importance. Their constant improvement makes it possible to reliably control the phytosanitary state of the planting material.

In June 2013, Official Monitor No. 136-139 Published Government Decree No. 415 on the approval of the Norm on the production, control, certification, and sale of material for the propagation and planting of fruit crops.

This document is harmonized with the legislative acts of the European Union and is a prerequisite for the integration of the Republic of Moldova into the European Union (Government Decision no. 390 of April 29, 1997; Government Decision no. 415 of June 21, 2013).

MATERIAL AND METHODS

Biotype of the clonal rootstock of the apple tree MM106 was used. The material of our research was the genetically resistant to scab *Venturia inaequalis* Cooke Wint 4 apple cultivars 'Coreor', 'Coredova', 'Coretard', 'Izvoras', obtained from the doctor habilitated professor Bukarchuk V.F. We used diagnostic kits for ELISA (Clarkand, 1977) to viruses of chlorotic apple leaf spot virus (ACLSV), apple stem grooving virus (ASGV), apple stem pitting virus (ASPV), apple mosaic virus (AMV). We also used diagnostic antisera of our production for the above viruses. Preliminary testing and retesting of cultivars were carried out using ISEM (Milne and Luisoni, 1977), ELISA, and the method of wood indicators. The ISEM results were visualized using a TESLA BS 500 electron microscope. The ELISA results were read using a SANOFY PR 2100 photometer.

Propagation of the clonal rootstock MM106 was carried out by the in vitro method using nutrient media in our modification. Explants 0.5, 2, 5 mm in size were isolated from artificially awakened buds in February, naturally swollen buds in March-April, and from the tops of shoots in May. The main nutrient medium was composed of macro and micro salts according to Murashige (1962), in our modifications. We changed the concentration of 6-benzylaminopurine from 1 to 4 mg/l, gibberellic acid from 0.5 to 1 mg/l. Rooting was made on agarized and liquid nutrient media with a reduced concentration of macro-micro salts, the absence of cytokinin, and varying the concentration of indole-3-butyric acid from 0.5 to 5 mg / L. The adaptation was carried out in various substrates consisting of peat, perlite, sand in different volume fractions. Plant acclimatization after in vitro was carried out in glass greenhouses during the spring period.

The production of diagnostic antisera to ASPV was carried out according to the methods established by other authors (Van Oosten, 1972; Yanase *et. al.*, 1989) and in our modification. We used 3 isolates of the ASPV found in the infected cultivars. Virus propagation was made on herbaceous indicator plants *Nicotiana occidentalis* 37B during the period necessary to obtain a purified virus preparation.

Dry-air thermotherapy was carried out in a specially designed thermal chamber located inside a glass greenhouse. The temperature in the daytime (four 16 hours) was $37\pm 2^{\circ}\text{C}$, and 28°C at night in the zone of the growth point (Chernets and Calashean, 2012). Heating was carried out using special equipment. The cycle of thermotherapy was carried out for 5

weeks. Grafting of cultivars tops was carried out on actively growing rootstock MM106, individually planted in plastic vessels.

RESULTS AND DISCUSSIONS

Micropropagation of MM106 rootstock

To develop a micropropagation technology, 10 mother bushes of a medium-sized MM-106 were used. The rootstocks were free of latent pome crop viruses. As a result of three years of experimentation, it was clear that the best time for isolation was March. The percentage of explants that started to grow was from 35 to 60% (Figure 1). To make nutrient media instead of 3 basic stock solutions, we used 7, which allowed us to use salt concentrates for a longer time (Chernets *et al.*, 2003). In this case, concentrated salt solutions were prepared using a special technique.

From the introduction into the culture to the stage of rooting of the plants, the rootstocks took from 7 to 10 passages. The average coefficient of formation of new shoots was from 1.2 to 4.56 per passage (Figure 2).

Figure 1. Plants after two months.

Figure 2. Mass micropropagation apple rootstocks.

Varying the concentration of thiamine chloride from 0 to 3.2 mg/L did not significantly affect either the number of new shoots or their height. An increase in the concentration of 6-benzylaminopurine (BAP) from 1 to 4 mg/L led to an increase in the number of additional shoots by 40%, but at the same time, the formation of callus on the bases of the shoots was noted, which is not desirable in the process of micropropagation.

At the stage of rooting, BAP was excluded from the composition of the medium and the concentration of macro and microelements was halved. From 80 to 100% rooting was obtained in variants with indole-3-butyric acid (IBA) 1-2 mg/l. The resulting rooted microplants had a height of 2.62-2.81 cm, 3-5 roots with a total length of up to 15 cm. As an alternative method of *in vitro* rooting, we used a liquid nutrient medium with a support made of special paper. The resulting plants were chlorotic with single small roots. An increase in the concentration of auxin led to the death of the tested plants.

One of the main stages in the technological process is the acclimatization of micropropagated plants grown under artificial conditions to the conditions of the greenhouse. Apple rootstock plants grown under aseptic conditions have an underdeveloped cuticle. The following adaptation factors are essential: substrate, air and substrate humidity, mineral fertilizers, temperature, pests, and diseases.

It is known that both low (+ 1-5°C) and high (+ 30-35°C) temperatures limit the survival rate of test tube plants. In the conditions of Moldova in unheated greenhouses, the optimal time for planting rootstocks according to the temperature factor turned out to be April-May. *In vitro* plants were planted in the evening.

The best substrate was a mixture of peat with perlite in a ratio of 1: 1 with a top layer of fine-grained perlite 3-4 cm high. The pH of the substrate was preliminarily adjusted to 6-6.2

by adding 15-25 g of lime dust per 10 kg of substrate. A mixture of perlite and peat retains moisture well, so it does not require frequent watering. But for successful adaptation, high air humidity was required, comparable to the air humidity in test tubes in which the plants were grown. We achieved this by placing plants under plastic wrap tunnels. The first 3 weeks after planting the relative air humidity was maintained at 80-90%. Then the humidity gradually decreased. Experiments conducted earlier showed that a sharp decrease in humidity leads to the so-called phenomenon of growth arrest, from which mini-plants can emerge only after passing through a period of winter dormancy.

Obtaining purified preparations of apple stem pitting virus

In the experiments on purification of received by us ASPV isolate, the methods developed in Holland by Nan Oosten (1972) and Yanase *et al.*, (1989) in Japan were taken as a basis.

In experiments where the technique developed by Japanese researchers was tested, the clarification of the plant extract was carried out with a gel consisting of dibasic sodium phosphate and calcium chloride. For this, systemically infected growing leaves of the indicator *Nicotiana occidentalis* 37B were homogenized in 0.02M phosphate buffer pH-7.4 in a ratio of 1:4. After low-speed centrifugation, the plant extract was added dropwise with constant stirring on a magnetic stirrer 1M Na₂HPO₄ in an amount of 6 ml for every 100 ml of vegetable juice and 1.5 ml/100 ml of 2M CaCl₂ under the same conditions.

The clarified preparation obtained after low-speed centrifugation was centrifuged on a 20% sucrose pad at 120,000 g for 90 min. The resulting precipitate was resuspended in 0.01M Na-phosphate buffer with the addition of 0.5% Triton X-100. After low-speed centrifugation, the preparation was ultracentrifuged on a 30% sucrose pad under the same conditions. The precipitate was resuspended in 0.01M borate buffer pH 8.0 with the addition of 0.5M urea to reduce particle aggregation. Checking in an electron microscope showed the presence of filamentous viral particles in the preparation. When determining the purity of the viral preparation from the admixture of normal proteins of the host plant using a spectrophotometer, we did not obtain a characteristic curve. Perhaps this was due to insufficient clarification of the plant extract at the initial stage of purification.

Taking into account the unsatisfactory results obtained by us in the purification of ASPV according to the method of Japanese researchers, we began to investigate the method of purification of the plum globule virus with approximate morphological parameters proposed by the Netherlands researcher Van Oosten, (1972). A significant difference between these methods lies in the fact that when developing a method for purifying the plum pox virus, used TRITON X-100 to clarify the plant extract.

After homogenization of the infected tissues, 3-5% TRITON X-100 was added drop by drop to the plant extract, which was gently stirred for 30 minutes on a magnetic stirrer at a temperature of + 40°C, followed by ultracentrifugation of the supernatant for 90 min at 90,000 g. The clarified plant sap was centrifuged for 10 min at 5000 g. Examination under an electron microscope showed the presence of numerous filamentous particles of various lengths in the specimen (Figure 3). However, attempts to fractionate the virus in a sucrose gradient of 10-40% were unsuccessful since the viral particles irreversibly precipitated.

In subsequent studies, we returned to the developments of Japanese researchers (Yanase *et al.*, 1989) with some changes that concerned the first stage of purification, namely, clarification of the plant extract. Instead of clarifying the plant extract with gel, TRITON X-100 was used. The clarified plant extract was ultracentrifuged at 90,000 g for 90 min. The resulting precipitate was suspended and centrifuged at 5000 g for 10 min to remove impurities.

Figure 3. ASPV particles before ultracentrifugation sucrose gradient. x18000

Figure 4. Purified preparation ASPV in a x18000

The previously purified virus suspension was further purified according to the method of Japanese researchers.

Inspection of the purified preparation in an electron microscope showed the presence of filamentous particles, the morphology of which was not disturbed (Figure 4).

The spectrophotometric curve of the purified preparation obtained by us, according to the optimized method, had one peak with a minimum at a wavelength of 240 nm and a maximum at a wavelength of 258 nm, which characterized the preparation as a nucleoprotein, (Figure 5).

Figure 5. The spectrophotometric curve of the purified ASPV preparation.

Max is indicated at a length of 258 nm.

The ratio of the A260 / 280 value is 1.5-1.8, which is consistent with the literature data. Such preparations of the purified virus were used to immunize experimental animals.

Antiserum preparation and determination of its quality

To obtain antisera to ASPV, we used a short immunization schedule, consisting of three injections of experimental animals with a weekly interval, and two reimmunizations at the time of falling to the minimum value of the antiserum titer. Chinchilla rabbits were selected for immunization. The first injection was performed using Freund's complete adjuvant, injecting the virus preparation subcutaneously along the spine. One week later, a virus preparation with incomplete Freund's adjuvant was injected into experimental rabbits into the large muscle of the hind legs. The third injection was carried out by injecting a pure virus preparation into the large veins of the rabbits' ears. On the 21st day from the start of immunization, blood was taken from the experimental animals from the ear vein to determine the activity of antibodies in the blood serum.

Determination of the quality of the antiserum was carried out by immunosorbent electron microscopy. For this, freshly obtained antiserum was diluted with 0.01 M Na/K - phosphate buffer pH-7.4 to values from 1: 8 to 1: 4096. Each fraction of the diluted antiserum was then used for making preparations in order to evaluate the activity of antibodies of the obtained

antiserum in an electron microscope. The criterion for evaluating the final dilution of the antiserum (titer) was the specific antigen-antibody reaction, which occurred during the treatment of virus particles caught on the object grid from the raw juice of infected *Nicotiana occidentalis* 37B plants with serum antibodies of the appropriate dilution.

Studies to determine the quality of antisera by the ISEM method with an interval of 7 days until the minimum value of antibody activity was reached. On the 62nd day from the start of immunization, the animals were boosted twice at weekly intervals. On day 77, the antiserum titer recovered to a value of 1: 2048. The above activity of antibodies of the obtained antisera was kept at a maximum level up to 115 days from the start of immunization.

The resulting high titer antiserum was used later to obtain a diagnostic kit for the studied virus and to optimize the ELISA.

The immunization scheme we used in the experiment made it possible to obtain a highly specific antiserum to ASPV.

Obtaining healthy clones by thermotherapy

Dry-air thermotherapy was carried out in special heat chambers developed by us in greenhouse conditions. In 2016, we tested all trees of the 'Coreor', 'Coredova', 'Coretard', 'Izvoras' cultivars growing in the institute's collection by the ELISA and ISEM method. These trees were tested for the presence or absence of viruses infecting the apple tree, which are mandatory for testing according to the certification system for virus-free planting material. Studies showed the presence of chlorotic apple leaf spot virus (ACLSV), apple stem pitting virus (ASPV) and apple stem grooving virus (ASGV) in all samples. As a result of our analyzes, the apple mosaic virus (AMV) was not detected. To carry out rehabilitation work in the summer of 2016, 15 plants of each cultivar were grafted. Buds for grafting were selected by the breeder author of the cultivars Bukarchuk V.F from trees that met all agrobiological and pomological characteristics of these cultivars. Within a cultivar, one tree was selected and assigned a special code. As a rootstock, we used a virus-free, medium-sized MM 106, which had been propagated earlier in vitro culture. The rootstocks were grown individually in three-liter vessels in an enriched soil mixture.

Table 1. Testing of apple samples by the ISEM after the first cycle of thermotherapy in 2018.

Cultivar	Number of clones obtained	ACLSV	ASGV	ASPV	AMV
'Izvoras'	7	0	1	1	0
'Coreor'	6	0	2	0	0
'Coredova'	5	0	0	1	0
'Coretard'	5	0	1	2	0

In 2018, 7 trees of the 'Coreor', 'Coredova', 'Coretard', 'Izvoras' cultivars were randomly placed in two heat chambers installed in a glass greenhouse in May. Within two weeks, the temperature in the growth zone of the apex was raised by two degrees every 48-72 hours. Thermotherapy was carried out for 35 days at a temperature in the chamber + 38°C ± 1°C and the temperature in the root zone + 28°C ± 2°C. The light period was natural (Figure 6).

In the course of thermotherapy, the death of individual plants was noted. We saw that 3-5 shoots developed from individual plants (Figure 7). From the shoots, the growth of which during the therapy was more than 35 cm, the tops of 0.5–1 cm in length were isolated. The tops were grafted onto a virus-free MM 106 growing individually in 1-liter containers. The maximum conditions were created for the best fusion of the tops. The survival rate varied from 35 to 40% depending on the cultivar. Two months later and in the spring of the following year, the plants were tested.

As a result of the first cycle of thermotherapy, we managed to heal the plants from the chlorotic leaf spot virus of the apple tree. (Table 1).

Apple stems pitting virus and apple stem grooving virus were identified as a result of testing.

Figure 6. Thermal chamber for carrying out Dry-air thermotherapy in greenhouse

Figure 7. Apple tree plant in thermal chambers at the stage conditions

Guided by the received data on the detection of latent viruses in individual plants it was decided to conduct a second cycle of thermotherapy in the next growing season. After the second cycle of dry air thermotherapy, the plants were tested again for viruses. The test results showed the absence of latent apple viruses (Table 2).

Table 2. Testing of apple samples by the ISEM after the second cycle of thermotherapy in 2019.

Cultivar	Number of clones obtained	ACLSV	ASGV	ASPV	AMV
'Izvoras'	8	0	0	0	0
'Coreor'	5	0	0	0	0
'Coredova'	6	0	0	0	0
'Coretard'	4	0	0	0	0

All plants that had undergone two cycles of dry-air thermotherapy were planted in a container with a volume of 10 liters (Figure 8). In 2020-2021the plants tested by ELISA using diagnostic kits from BIOREBA AG and LOIWE showed the absence of viruses. Biological testing on woody indicators showed the absence of viruses.

The more developed two-plant cultivars category Prebase were planted in the depositarium. In depositarium of this kind, the trees are kept following international standards for the certification of virus-free planting material. The presence of a mosquito net prevents the ingress of insects, vectors-carriers of viral diseases.

In the future, the plants will be propagated according to certification schemes European and Mediterranean Plant Protection Organization.

Figure 8. Apple plants that undergone two cycles of thermotherapy.

CONCLUSIONS

The technology of micropropagation in vitro of vegetative medium-sized rootstock MM106 has been worked out. The diagnostic polyclonal antiserum obtained by us to ASPV showed sensitivity at the level of diagnostic kits to this virus from other manufacturers. The adapted plants were used for laying a mother plant in the field and for carrying out experiments on the improvement of scab-resistant apple 'Coreor', 'Coredova', 'Coretard', 'Izvoras' cultivars. The use of two cycles of thermotherapy made it possible to obtain completely virus free clones of cultivars. The trees category Prebase was transferred to depozitarium (nuclear stock) according to certification schemes European and Mediterranean Plant Protection Organization.

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