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FINAL TECHNICAL REPORT
1.9.91 - 1.9.94

IN VITRO BIOLOGICAL INDEXING IN FRUIT TREE QUARANTINE SYSTEM

Project Number: C-11-065

Shmuel Zilkah (Israel) and Barbara Zawadzka (Poland)

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General Conclusions

Background

The overall aim of the project was to examine the hypothesis of using *in vitro* methodology for speeding the fruit tree quarantine systems.

The main objective was planned to be addressed through several steps:

1) To develop *in vitro* methodology for culturing apple and peach varieties.

2) To establish peach and apple cultures healthy and infected with known viruses.

3) To prepare *in vitro* cultures of specific plant indicators to peach and apple diseases

4) To develop micrografting methods enabling virus transmission to indicators

5) To manipulate the media and environmental conditions for induction of clear symptoms on infected cultures.

In the following report we are covering the progress and the achievement made in the various research steps in peach (Israel) and apple (Poland) systems.

1. PEACH SYSTEM

1.1. Establishment of *in vitro* virus population of selected cultivars

At the first stage of the project efforts have been concentrated on development of a culturing medium which will be common to as many as possible, of peach (*Prunus persica* L.) varieties.

Among several basal media that been tested, the most suitable basal medium for peach varieties was Almehdi and Parfitt (AP) medium. For the initial stage of the explant establishment, the hormone composition of 0.2 mg/l BA + 0.01 mg/l IBA was the most suitable. For proliferation and elongation stages higher levels of hormones were necessary (6 mg/l BA + 0.01 IBA). Half strength of MS medium + 0.5 mg/l NAA was used for rooting. This basic protocol with minor modification was suitable to various peach cultivars such as Hermosa, Babcock and Summerset.

Tissue cultures with different level of PNRSV infection have been prepared from buds initially infected with gradual levels of virus or through virus elimination by using thermotherapy and shoot tip culturing. The infected and virus free clones have been used for virus transmission experiments.

The list of peach virus source cultivars has been expanded to sweet cherry (*P. avium*) cvs. 40 E 50 and 106 EB 325. Virus free clones of them have been also established by using *in vitro* thermotherapy (Stein et al. 1991) and shoot-trip culturing. Plants were regenerated and reindexed. By this methodology it was possible to introduce, in a relatively short time new imported sweet cherry cultivars to commercial plantations in Israel. Summerizing paper (Zilkah et al., 1994) is hereby enclosed.

One of the controls for virus infected plants which is rountinely used in quarantine indexing process, is the almond (*P. amygdalus* Batch) cv. Hazanov. Tissue culture of this cultivar was developed. Virus-free clones of this cultivar have been also prepared through the mentioned methodology. They have been used for micrografting and symptom induction studies.

Serological ELISA procedures for detecting the Hermosa tissue culture infected with various levels of PNRSV were optimized. Experiments have been carried out to intensify the ELISA method sensitivity for detecting virus infection in a very low level. All treatments such as, manipulation of NH_4^+/NO_3^- ratio in medium, application of moderate stress by using urea and the plant growth retardant paclobutrazol and incubation at different light and temperature conditions, have not exhibited a significant improvement of ELISA detection sensitivity to PNRSV.

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Rapid introduction of early season sweet cherry cultivars through *in vitro* micropropagation and virus elimination

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Key words: Prunus avium, quarantine, shoot culture, tissue culture

Summary

In vitro propagation techniques were used for the rapid introduction of new imported sweet cherry (*Prunus avium* L.) cultivars, 17 H 580, 40 E 50 and 106 EB 325. Media for bud establishment, bud shoot proliferation, shoot elongation and rooting were developed for each of them. The cultivars differed in their response to different media compositions. Indexing on several plant indicators under quarantine conditions found the cultivars 40 E 50 and 106 EB 325 to be virus infected. *In vitro* thermotherapy in combination with shoot-tip culturing was successfully applied. Virus-free cultures were rooted, acclimated and grown under greenhouse and field conditions.

Abbreviations: BA – 6-benzylaminopurine; GA_3 – gibberellic acid; IBA – indole-3-butyric acid; NAA – naphthaleneacetic acid

Introduction

One of the most important objectives for improvement of the sweet cherry industry in Israel is the extension of the marketing season. Early season cultivars have an economic importance for the development of new domestic and export markets. Sweet cherry (*Prunus avium* L.) cultivars 17 H 580, 40 E 50 and 106 EB 325 were selected by Zaiger's Inc. Genetics, USA. They were imported to Israel for potential use as early season cultivars.

Sweet cherry rootstocks and scion cultivars have been found difficult to propagate using hardwood cuttings (Feucht & Dausent 1976, Hartman & Brooks 1976). *In vitro* techniques have the potential to shorten significantly the time necessary for introduction of new imported plant varieties. Micropropagation could be performed concomitant to the indexing procedures of the quarantine station. Adequate proliferating material could be prepared *in vitro* and be readily used after certification of the cultivar for distribution. When virus infected material is identified by the quarantine procedures, *in vitro* heat therapy (Snir & Stein 1985, Stein et al. 1991) and shoot-tip culture could be used for virus elimination. In previous studies, cherry rootstocks (Jones & Hopgood 1979, Jones et al. 1984, Zilkah et al. 1992) as well as sweet cherry scion cultivars (Snir 1982a,b, Zilkah et al. 1993) were successfully micropropagated. The cultivars in the present study were new and had to be evaluated.

The present paper deals with the multiplication by tissue culture of three new sweet cherry cultivars to enable their rapid introduction.

Materials and methods

Cuttings with dormant buds were collected in December from one-year- old twigs of sweet cherry cultivars 17 H 570, 106 EB 325 and 40 E 50. The cuttings were incubated at 4 °C for 2–4 months until bud swelling. Short segments containing 1–2 buds were surface-sterilized by stirring in 80% isopropanol for 30 min. followed by rinsing with sterile distilled water. Each bud was dissected separately and transferred immediately to reducing solution containing 100 ppm dithio-

threitol. Shoot-tips, about 1 mm length, including meristem-tip and several leaf premordia, were taken for culture initiation in 10 × 2.5 cm test-tubes, containing 10 ml c culture medium each and closed with plastic caps (B .co-Kaput). The cultures were placed in a controlled environment room, with 16 h photoperiod at an irradiance intensity of 3 W/m^2 in the visible range and at a relative humidity of 50- 60% at 25 °C.

Immersion of cv. 40 E 50 culture in a solution containing 50 mg/l rifampicin for 24 h significantly suppressed the endophytic bacterial contamination. When temporary treatments were not effective enough, some clones of this cultivar were continuously cultured on medium supplemented with 250 mg/l carbenicillin.

Virus elimination has been carried out through heat therapy (Stein et al. 1991) followed by shoot-tip culturing.

Results

Initiation and establishment

Dissected buds were transferred to different media already used for initiation of sweet cherry rootstocks (Zilkah et al. 1992). Tabachnic & Kester medium (Tabachnic & Kester 1977) supplemented with the growth regulators 1 mg/l IBA and 1 mg/l BA gave the best results for all three cultivars. Of the buds cultured for cvs 17 H 580, 106 EB 325 and 40 E 50, 14, 60 and 46%, respectively, developed to form a rosette with several leaves during the first 4–6 weeks in culture.

Proliferation, elongation and rooting

Established cultures were transferred to shoot proliferation media. Media were based on Murashige & Skoog (MS) medium (Murashige & Skoog 1962) and Almehdi & Parfitt (AP) medium (Almehdi & Parfitt 1986) with various combinations of IBA, BA and GA_3concentrations. The three cultivars responded differently to medium conditions. Cv. 17 H 570 proliferated at a rate of 3 shoots per subculture on MS medium supplemented with 1 mg/l IBA, 1 mg/l BA and 0.5 mg/l GA_3. On this medium, relatively elongated shoots were developed. No other medium for elongation was necessary and the cultures could be transferred directly to the rooting medium. The best rooting rate (> 90%) was obtained when the shoots were separately cultured on half strength MS medium containing 0.5 mg/l NAA.

In response to the same proliferating and elongating medium, cv. 106 EB 325 developed relatively elongated shoots but multiplied at a lower rate (2–2.5 per subculture at 4–5 weeks intervals). Shoots from this stage could also be transferred directly to rooting medium. For mass multiplication, however, shoots of cv. 106 EB 325 were transferred to AP medium containing 3.5 g/l agar, 1 g/l Gelrite, 0.01 mg/l IBA, 0.5 mg/l BA and 0.2 mg/l GA_3. The multiplication rate in this medium was > 3.5 shoots per subculture. However, the shoots did not elongate satisfactory (Fig. 1) and single shoots had to be transferred to MS elongating medium containing 1 mg/l IBA, 1 mg/l BA and 0.5 mg/l GA_3. High rooting rate (> 90%) was obtained by using half strength MS medium containing 0.5 mg/l NAA.

In contrast to the other two cultivars, shoots of cv. 40 E 50 could not be multiplied or elongated on MS medium. AP medium containing 3.5 g/l agar, 1 g/l Gelrite, 0.01 mg/l IBA, 0.5 mg/l BA and 0.2 mg/l GA_3 was a suitable proliferating medium (2.5–3 per subculture), but only about 20% of the developed shoots could be rooted. For elongation, the shoots were transferred to AP medium containing 0.01 mg/l IBA, 0.2 mg/l BA, 3.5 g/l agar and 1 g/l Gelrite. Over 90% of the shoots then developed sufficiently for root induction.

Cv. 40 E 50 was difficult to root. Various combinations of MS medium and the conventional rooting hormones NAA, IAA and IBA were tested. Fewer than 10% of shoots produced roots. IAA conjugates, which have been shown to act as rooting compounds (Wiesman et al. 1989), were tested too but with no significant success. After > 20 subcultures 40 E 50 started to form roots at an increasing rate (up to > 85%) on half strength of MS medium supplemented with 1 mg/l IBA. This medium gave better results than the rooting medium used for the other cultivars tested.

Virus elimination

During the development of the micropropagation methodology, the cultivars were indexed under the official quarantine procedure. Cv. 106 EB 325 was found virus-infected on *Prunus serrulata* cvs. Shirofugen and Kwanzan indicators. Cv. 40 E 50 were found infected on the latter indicators and also on *P. avium* cv. Bing and *P. persica* cv. Elberta. Cv. 17 H 580 was found infected with prunus necrotic ring spot virus detected by enzyme-linked immunosorbent assay (ELISA) as previously described (Snir & Stein 1985). *In vitro* thermotherapy in combination with shoot-tip culturing was applied. Plants were regenerated and indexed, and

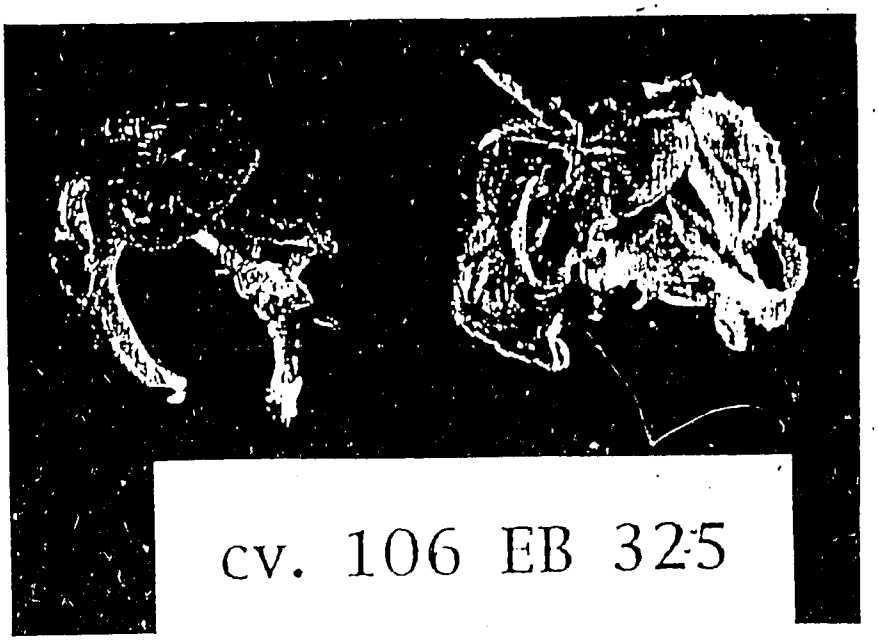

Fig. 1. Shoot culture of sweet cherry cv. 106 EB 325 grown on a single stage multiplication/elongating MS medium containing 1 mg/l IBA, 0.5 mg/l BA and 0.5 mg/l GA_3 (left). Same culture grown on multiplication AP medium containing 0.01 mg/l IBA, 0.5 mg/l BA and 0.2 mg/l GA_3 (right).

those found to be virus-free were clonally propagated. They were distributed as mother plants to nurseries and used in trial plots as a part of the introduction procedure.

Discussion

Although sweet cherry cultivars are difficult to propagate from hardwood cuttings (Feucht & Dausent 1976, Hartman & Brooks 1976), tissue culture methodology has opened new possibilities for proliferation of rootstock (Jones & Hopgood 1979, Jones et al. 1984, Zilkah et al. 1992) and scion (Snir 1982a,b, Zilkah et al. 1993) cultivars. Tabachnik & Kester medium gave the most satisfactory results for shoot establishment from buds for all the cultivars used in this study and also for other *P. avium* (Snir 1982a,b) or *P. avium* × *P. mahaleb* (Zilkah et al. 1992) cultivars. Endophytic bacterial contamination was a severe problem in establishing shoots from cv. 40 E 50 buds. No phenological variants were observed amongst plants derived from cv. 40 E 50 cultures grown on antibiotics.

Cultivar 40 E 50 was difficult to root. Only after > 20 subcultures was significant rooting obtained on a standard medium composition of 1/2 MS strength + 1 mg/l IBA. This phenomenon has been observed in other difficult to root species such as almond (Rugini & Verma 1982/83), apple (Sriskandarajah et al. 1982) and another early season sweet cherry cultivar, Burlat (unpublished data). We recently reported that difficult to root 40 E 50 cultivar was significantly different in IBA metabolism than easy to root cultivar (Epstein et al. 1993).

It is important to emphasize that the micropropagation procedures and thermotherapy treatments could be done routinely concomitant to the indexing procedures taken in the quarantine station. Certified cultivars officially released for distribution, could be immediately available for large-scale preparation of mother-plants for experimental and semi-commercial trials. These tissue culture approach is most advantageous for rapid introduction of difficult to propagate rootstocks such as sweet cherry (Zilkah et al. 1992). If the indexing and micropropagation were reliably done by the exporting country, then the movement among countries of new germplasm as pathogen-free tissue cultures would be much easier and faster.

Conclusions

Micropropagation methodologies have been developed for three potential early season sweet cherry cultivars concomitantly to the indexing procedures taken in quarantine. The *in vitro* methodology enabled rapid production of virus-free plants for testing in introductory experimental plots and release to nurseries.

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1.2. Development of *in vitro* indicators

Tissue culture of three indicators have been developed and selected for micrografting suitability. *P. persica* cv. Elberta, *P. serrulata* cv. Shirofugen and *P. tomentosa*. An article summarizing the micropropagation methodology of the developed indicators (Zilkah et al., 1993 Acta Horticulturae 336: 121-125) is hereby enclosed.

IN VITRO MICROPROPAGATION OF INDICATOR PLANTS FOR INDEXING PRUNUS NECROTIC RING SPOT VIRUS

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Additional index words: Shirofugen (*Prunus serrulata*), *Prunus tomentosa*, Elberta (*Prunus persica*), tissue culture, quarantine

Abstract

The objective of the present study was to develop an *in vitro* system for propagation of plant indicators to Prunus necrotic ring spot virus (PNRSV) as a model for the purpose of shortening the time of indexing. Three plant indicators were micropropagated: *Prunus persica*, cv. Elberta, *P. tomentosa* and *P. serrulata*, cv. Shirofugen. The medium for shoot culture establishment were Almehdi and Parfitt (AP) containing 0.2 mg/l BA and 0.01 mg/l IBA,for both *P. tomentosa* and Elberta, and Boxus medium containing 1 mg/l BA, 0.01 mg/l GA_3 and 0.02 mg/l 2,4-D for Shirofugen. The media for shoot proliferation were AP + 0.01 mg/l IBA, 0.5 mg/l BA and 0.2 mg/l GA_3 for both Shirofugen and Elberta and AP+0.01 mg/l IBA and 0.2 mg/l BA for *P. tomentosa*. The rooting medium was half strength Murashige and Skoog basal medium containing 0.5 mg/l NAA for both *P. tomentosa* and Elberta and 1.0 mg/l IBA for Shirofugen. PNRSV infected peach cultures were reciprocal grafted in sterile conditions on indicators. Virus transmission to the indicators has been detected by ELISA method.

1. Introduction

Exchange of plant material among countries is always connected with the possibility of introducing diseases which may be devastating for commercial fruit industries. When vegetatively propagated material from wild and exotic sources is introduced, length observations and indexing under quarantine conditions are required. Indexing for viral and virus like disease (VLDs) is achieved by grafting buds onto sensitive indicator plants and observing symptoms. This procedure may require up to several years and thus become a severe impediment in new germplasm movement.

The *in vitro* technique may offer potential improvement for quarantine. It is extremely space saving, less contaminated with exogenous pathogens and more amenable to manipulation of environmental conditions for improving the survival of the plant material and the evaluation of its virus infection status.

Shirofugen (*Prunus serrulata*) cherry, *P. tomentosa* and Elberta (*P. persica*) are hosts which are commonly used for indexing of PNRSV (Anon, 1976). The objective of the present paper is to describe the micropropagation system for three virus indicator plants as a step in

developing rapid *in vitro* indexing.

2. Materials and methods

Seeds of *Prunus tomentosa* were disinfected and each one was sown into 50 ml vessels containing mixture of peat moth: polystyrene foam (2:1). The seedlings raised in a growth chamber illuminated with a 16 hr photoperiod, irradiance intensity of 2000 $\mu W/cm^2$, relative humidity of 60-70% at 26°C. When the seedlings reached 7-8 cm long they were transferred to 500 cm^3 pots and raised at the same conditions up to the height of 60-80 cm. Explants, 3-5 cm long were collected from the actively growing shoot tips. The explants were prepared by removing the leaves (except of part of the petiole, 5 mm long, which was left in each leaf), soaking them for 2 minutes in a solution of 0.04% sodium hyperchloride and 0.01% Tween 20 and 3 times washing in sterile distilled water. The explants were left for 24 hr in 0.7% agar medium. Then, they were removed, washed with sterile distilled water for 15 minutes, stirred in a solution of 0.15% sodium hypercloride and 0.25% Tween 20 and 3 times washed in sterile distilled water. The explants were cultured on various types of solid media.

Cuttings from one-year old twigs of Shirofugen and Elberta were kept at 4°C. When the buds started to swell the cuttings were cut into short segments and surface disinfected by washing for 30 min in 80% isopropanol, followed by rinsing with sterile water. Immediately after excision, each segment was submerged in a solution of a 100 ppm dithiothreitol as a reducing agent. The tips of the dissected buds (about 1 mm), inlcuding the meristem-tip and several leaves, were placed in 2.5 x 10 cm test tubes which were closed with plastic caps (Bellco-Kaput). The test tubes contained various media for initiation and establishment of shoot tissue culture.

3. Results

3.1. Initiation and establishment

Several different media have been tested. The best medium for shoot culture establishment of *P. tomentosa* and Elberta was Almehdi and Parfitt (AP) medium (Almehdi and Parfitt, 1986) containing 0.2 mg/l BA and 0.01 mg/l IBA. The optimal medium for initial establishment of the Shirofugen buds was Boxus medium (Boxus and Quoirin, 1974) containing 1 mg/l BA, 0.1 mg/l GA_3 and 0.02 mg/l 2,4-D. The test tubes were incubated for several weeks in growth chamber with the conditions described in Materials and Methods. When Shirofugen bud tips formed a rosette with several leaves and the Elberta and *P. tomentosa* explants were in active growth, the cultures were transferred to various media for multiplication.

3.2. Multiplication and growth

After testing several basal media with different hormone combinations the following media was found to be most suitable: AP medium containing 0.01 mg/l IBA, 0.5 mg/l BA and 0.2 mg/l GA_3 for

Shirofugen and Elberta. For *P. tomentosa* the hormone composition of 0.01 mg/l IBA and 0.2 mg/l BA in AP medium was more suitable. The multiplication factors were 4-5, 3-4 and 1-2 per subculture and the subculturing intervals were 6-7, 8-9 and 4-5 weeks for *P. tomentosa*, Shirofugen and Elberta, respectively.

3.3. Rooting

Best rooting for *P. tomentosa* (100%) was obtained with half strengh of Murashige and Skoog basal medium (Murashige and Skoog, 1962) containing 0.5 mg/l NAA. For Shirofugen the same basal medium with 1 mg/l IBA gave lower rate of rooting (50%) than with 0.5 mg/l NAA but the quality of the obtained roots was significantly better. Elberta was initially rooted in 0.5 MS + 0.5 mg/l NAA medium. The explants which failed to root at the medium were rooted eventually after transferring them to Jiffy-7 pellets soaked with water.

3.4. Hardening

Rooted plantlets were transferred to peat pellets (Jiffy 7) and maintained until the roots emerged from the pots. The humidity was kept at >90% by enclosing the Jiffy pellet with transparent boxes (Magenta). The plantlets with the Jiffy were transferred to 500 ml pots with peat-compost mixture (1:1) and kept under 500 $\mu W/cm^2$ light. The humidity was gradually reduced and the plants were taken outdoors to a screenhouse.

3.5. Preparation of the plant indicator tissue cultures for successful micrografting

In order to be able to carry out a successful transmission of virus to the indicator's tissue cultures, suitable conditions for reciprocal micrografting between tissue cultures of the virus sources and indicator's tissue cultures had to be developed. An important factor for obtaining a successful micrografting is the stem thickness. Its diameter has to be adequately large in order to get a good contact between the vascular rings of the scion and rootstock.

3.5.1. Selection for an appropriate *P. tomentosa* clone

The tissue cultures clones that have been obtained from different seedlings of *P. tomentosa* had a significant diversity in respect to multiplication capacity (number of shoots which were developed from a single shoot culture) and the stem thickness (shoot diameters ranged between 0.3-2.0 mm) (Fig. 1). These two traits were negative correlated in a significant manner (Fig. 2). It seems that the developing shoots in the same culture are in a mutual competition. The clonal variation was exploited for selecting a thicker stem clone. This population of the thicker clones was used for further selection through application of paclobutrazol (a retardant plant growth regulator). Paclobutrazol was applied in the medium prior to autoclavization. It reduced significantly the shoot proliferation and growth. On the other hand, paclobutrazol induced callus formation on the basipetal end of the culture (Fig. 3). From the cultures that were exposed to 0.2 ppm paclobutrazol it was possible to select, after

3-4 cycles of subculturing on free paclobutrazol medium, a clone with significant higher rate of cultures suitable to micrografting.

3.5.2. Selection for an appropriate Shirofugen clone

The Shirofugen cultures that were obtained originally from bud dissection culturing had a relative good multiplication rate (factor of 3-4 per subculture). However, the growth was relatively slow. Plants were regenerated from the original tissue cultures and were raised vigorously. When they reached 60-70 cm height, 5 cm of their shoot tips were removed to agar medium containing the composition of the original establishment medium for Shirofugen. The cultures that were developed from the shoot tips of the *in vitro* propagated plants had significantly better growth rate and larger stem width which made them more suitable to micrografting.

4. Discussion

The goal of the present study was to develop a *in vitro* micropropagation system for three PNRSV indicator plants. The cultures were selected for clones with vigorous growth and appropriate stem which made the cultures more amenable to micrografting with the infected tissue cultures. The intention to use *P. tomentosa* as an indicator led to develop alternative conditions to *in vitro* propagation that had been already published for this species (Norton and Boe, 1982). It seems that the goal of this study was fulfilled. Selected clones of indicators suitable to virus transmission through micrografting are being efficiently micropropagated. Actually, PNRSV infected peach cultures were reciprocal grafted in sterile conditions with the developed indicator cultures. Virus transmission to the indicators has been detected by ELISA method (detailed data not shown).

The developed method for rapid propagation of the selected clones can be used also for traditional virus indexing. It is possible to perform the indexing in the greenhouse all year round. Detection of PNRSV symptoms, by *in vitro* generated Shirofugen plants has already been done by us (unpublished data).

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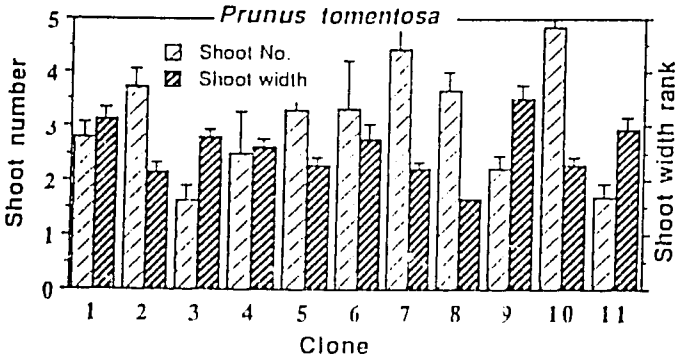

Figure 1. Variation among clones of tissue cultures derived from different seedlings of *Prunus tomentosa*. Two traits were evaluated: multiplication rate (number of shoots that were developed from a single shoot in one cycle of subculturing) and the shoot width. The shoot width (diameter) ranged between 0.3- 2 mm (valued into 1-5 degrees). Each column represents an average of 5-24 cultures per clone.

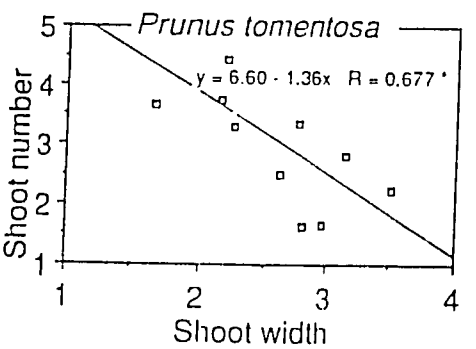

Figure 2. Correlation between shoot number and shoot width among tissue culture clones derived from *P. tomentosa* seedlings. Coefficient is significant α =0.05%

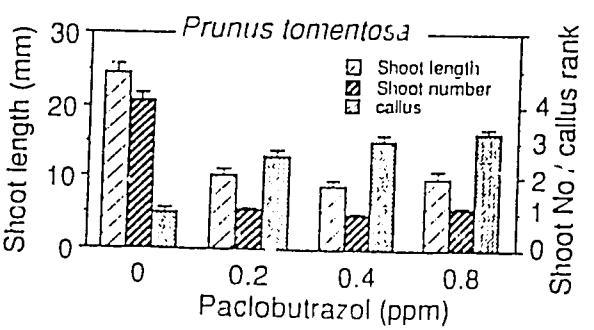

Figure 3. Effect of paclobutrazol on shoot length, shoot number and callus formation of *Prunus tomentosa* tissue culture.

1.3. Micrografting and virus transmission

Several micrografting methods have been screened for graft taken optimization. Stem diameter <1.5 mm was a limiting factor for graft success. Therefore, selection for thicker stem was made (i.e. cv. Tomentosa). Scion stem length and leaves amount were also important factors. Top grafting was compared to sideway grafting. The highest rate of graft taken (>90%) was obtained when scion stem (10-15 mm in length; leaves were cut at the blade basis) were wedge grafted onto split stock without any sealing. Micrografting was carried out out of tube, under saturated humidity. Cvs. Hermosa and Elberta segments infected with PNRSV was grafted as a scion on Shirofugen and Elberta indicators which have been used as a rootstock and vice versa. Virus transmission was detected by ELISA.

Since tissue cultures are heterothrophic and nutrient transport is mostly acropetally, indicators were usually grafted as scions. No significant symptoms were visible on Elberta indicator when cvs. Hermosa, Sumerset or 40 E 50 were used as virus infected and virus-free stocks. When Shirofugen (*P. serrulta*) was grafted onto cvs. Summerset or Hermosa (*P. persica*), necrosis was developed on the union site of both virus infected and virus-free stocks, pointing out on scion/stock incompatibility. Necrotic symptoms have been developed when Shirofugen has been grafted onto virus infected Elberta indicator, sweet cherry cv. 40 E 40 and almond cv. Hazanov. Such necrotic responses were not shown when virus free controls were grafted. These results show that successful indexing could be done also on the tissue culture level indicators. However, the *in vitro* indexing system i.e. indicator responses, viruses and plant species has to be recalibrated.

A paper describing the symptom results on cv. Shirofugen is enclosed by (Zilkah et al., 1995, Acta Horticulturae, in press).

SYMPTOMS OF PRUNUS NECROTIC RINGSPOT VIRUS ON MICROGRAFTED PRUNUS SERRULATA CV. SHIROFUGEN SHOOT CULTURES

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ABSTRACT

Prunus serrulata cv. Shirofugen is used worldwide as a biological indicator for virus diseases of Prunus spp. Indexing is performed traditionally by graft inoculations onto fully-developed Shirofugen plants which are then observed over a long period of time for symptoms. In the present study, in vitro indexing was attempted with micropropagated Shirofugen shoots and prunus necrotic ringspot virus (PNRSV)-infected shoot cultures of peach (cvs. Hermosa and Elberta) and sweet cherry (cv. 40 E 50). Micrografting was done in vitro in two ways: (a) Shoot cultures of the infected cultivars were used as 'rootstocks' onto which Shirofugen shoots were micrografted as 'scions'. (b) Shirofugen serving as 'rootstocks' and cultivars as 'scions'. Healthy cultivars were included for controls. Preliminary results showed that a typical symptomatic necrosis developed when Shirofugen was grafted as a 'scion' onto PNRSV-infected Elberta peach and 40 E 50 sweet cherry and did not develop with healthy controls. These results are preliminary. To develop a reliable in vitro indexing system, additional work aimed to improve graft survival and reduce non specific reactions is necessary.

INTRODUCTION

Indexing for fruit tree viruss is performed traditionally by graft inoculation onto fully-developed woody indicator plants which are then observed for symptoms over a period of several months at least. This long procedure is labor-intensive and often season dependent. Greenhouse indexing with woody indicators allows a considerable shortening of the period of time required for symptom development (Fridlund, 1980, Gilles and Bormans, 1988). In vitro indexing follows the same basic principal, but with indicators and shoots to be tested grown on a nutrient medium and micrografted under aseptic conditions. It has several potential advantages over the traditional procedure: it is amenable to manipulation of medium components and other environmental conditions, free of exogenous pathogers, requires a relatively small space, and enables rapid preparation of plant indicators through micropropagation procedures. The apparent prerequisite for a successful in vitro indexing system is the ability to induce on the indicators visible symptoms specific to virus infection .

In this study, a model system consisting of Prunus serrulata

cv. Shirofugen - a biological indicator used worldwide for virus diseases of Prunus spp. and shoot cultures of peach and sweet cherry cultivars infected with prunus necrotic ringspot virus (PNRSV) were selected for evaluation of the reliability of in vitro indexing.

MATERIALS AND METHODS

Tissue cultures: PNRSV-infected and healthy culture clones of three Prunus cultivars have been developed, grown and routinely subcultured on adopted media compositions. Peach (P. persica L.) cvs. Elberta and Summerset were grown on a medium (AF, following Almehdi and Parfitt (1986) supplemented with the hormone composition of 0.2 and 6.0 mg/l benzyl aminopurine (BA) for elongation and proliferation stage, respectively; and 0.01 mg/l indole benzoic acid (IBA). Sweet cherry (P. avium L.) cv. 40 E 50 was cultured according to Zilkah et al., (1994). The plant indicator P. serrulata cv. Shirofugen was cultured according to Zilkah et al., (1993). Shoot cultures of peach and cherry were tested by ELISA to confirm systemic infectivity with PNRSV.

Micrografting: Shoot cultures grown on an elongation medium were used for micrografting which was performed in two ways:

a. PNRSV-infected shoots were used as 'rootstocks' onto which Shirofugen shoots were micrografted as 'scions'.
b. Shirofugen shoots were serving as 'rootstocks' and the cultivars as 'scions'.

For controls, healthy shoot cultures of the respective cultivars and indicators were micrografted as described above.

RESULTS AND DISCUSSION

The grafted indicator - cultivar combinations, performed by the two ways described in Materials and Methods, were observed over a period of 4 weeks after grafting. A typical necrotic reaction was observed on Shirofugen shoots grafted as 'scions' onto infected peach and sweet cherry cultivars serving as 'rootstocks' and did not occur with the respective healthy controls (Fig. 1). No reaction occured when infected cultivars were grafted as 'scions' onto Shirofugen shoots serving as 'rootstocks'(not shown). These results suggest a possible association of the symptoms observed on the 'scion' with the movement of nutrients from the medium to the tips of shoots grown in vitro.

Table 1 summarizes a typical indexing experiment in which graft survival and expression of symptoms on Shirofugen grafted onto sweet cherry and peach were evaluated. Single shoots grown on an elongation medium prior to- and following grafting were suitable for micrografting. Graft survival depended on age (time after subculture to a fresh medium), shoot diameter and firmness of both

indicator and host. A difference was observed in graft survival and numbers of positive/tested samples of the two peach cultivars. This is probably due to the relative thinness of Summerset shoots which may have affected contact between the grafted indicator and the host shoot tissue. These results are preliminary. To develop a reliable in vitro indexing system, additional work aimed to improve graft survival and reduce non specific reactions is necessary.

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Table 1 - *In vitro* indexing for PNRSV by micrografting Shirofugen shoots onto sweet cherry 40 E 50 and peach Elberta and Summerset shoot cultures

Source Plant Material	No. of Shoots Micrografted	Graft survival	P/T[1]
Sweet cherry 40E50			
Infected	30	29 (97)[2]	24 / 29 (83)[3]
Healthy	19	17 (90)	2 / 17 (12)
Peach Elberta			
Infected	30	23 (77)	22 / 23 (96)
Healthy	29	27 (93)	2 / 27 (12)
Peach Summerset			
Infected	34	23 (68)	17 / 23 (74)
Healthy	36	22 (61)	4 / 22 (18)

In vitro propagated Shirofugen shoots were grafted onto shoots of sweet cherry (cv. 40 E 50) and peach (cvs. Elberta and Summerset) grown on a nutrient medium and observed for 30 days for symptoms. Graft survival was evaluated visually 10 days after grafting. Typical necrosis on Shirofugen shoots was observed 14-23 days after grafting. [1]number of positives vs tested. [2]percent surviving grafts. [3]percent positives vs tested.

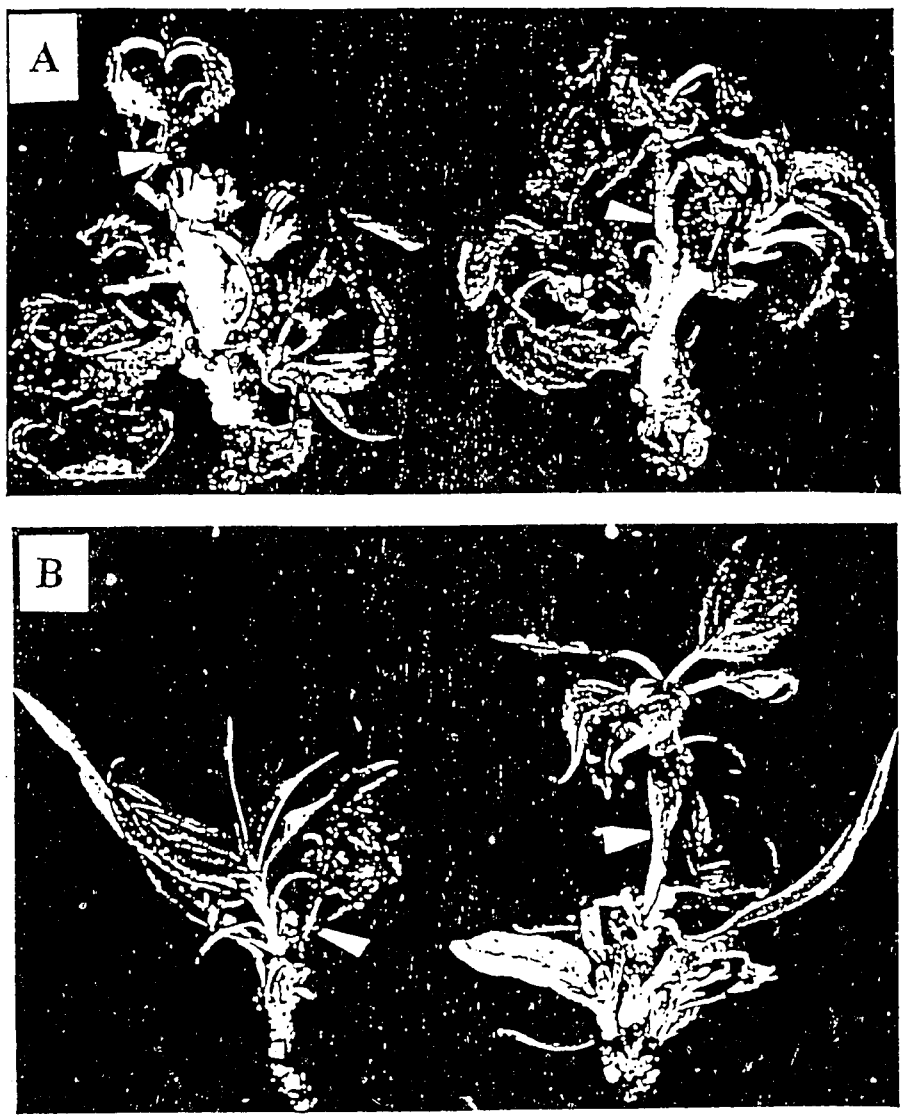

Fig. 1 - Necrotic reaction on Prunus serrulata cv. Shirofugen micrografted onto sweet cherry cv. 40 E 50 (A) and peach cv. Elberta (B) infected with prunus necrotic ringspot virus (left) and non-infected controls (right). Arrow heads indicate micrografted Shirofugen shoots.

1.4 Use of micropropagated indicator plants for virus indexing

In vitro micropropagation of indicator plants enable mass production of selected clones that give the best symptom response in the indexing process. Indicators, at their stage of development which gives the optimal response, could be produced all year around. We found that micropropagated Shirofugen could be successfully used for indexing when already 10 cm plants, were grafted by virus source of either woody buds (cvs. Hermosa, Hazanov, 40 E 50) or tissue cultures (cv. 40 E 50). This outcome of the project could be immediately applied to the routine indexing process.

1.5 Manipulation of media and environmental conditions for symptom induction

Since no symptoms were visible on leaves of Elberta or Tomentosa indicators, it has been hypothesized to induce symptom expression under mild stress conditions. Several nutritional and environmental factors have been manipulated to exert such stresses.

Sucrose has been replaced by lactose, sorbitol, manitol, galactose or glycerol. Effect of ABA, urea, paclobutrazol on symptom expression were also examined. Effect of diluted medium, hormone composition, pH, temperature and light on infected Elberta and Tomentosa indicators, and on infected cvs. Hermosa, 40 E 50 and 106 EB 325 were all tested. Although, in some cases virus infected tissues were found to be more sensitive than virus free ones, the results could not be routinely reproduced.

2. APPLE SYSTEM

In the research on suitability of in vitro micrografting of indicators to check apples for virus infection, apple chlorotic leaf spot virus (ACLSV) had been chosen as a model pathogen. However, two other viruses and viruslike pathogens were also considered: apple stem pitting virus and apple proliferation MLO.

The proper indicators used for detecting and identification of those pathogens were also micropropagated.

2.1. Material and methods

2.1.1. Choice of plant material and development of procedures for in vitro propagation.

The following indicator plants were taken for experiment with propagation in vitro:

1. Russian Seedling R 12740-7A and Malus platycarpa
   - for detection of ACLSV (Mink, and Shay, 1959).
2. Golden Delicious - for detection of apple proliferation (MLO) (Kunze, 1989).
3. Virginia Crab - for detection of apple stem pitting virus (ASPV) and apple stem grooving virus (ASGV) (Millikan, and Guengerick, 1959).
4. Spy 227 - for detection of Spy epinasty and decline (probably the same pathogen, which causes ASPV) (Stouffer, 1989).
5. Lord Lambourne - for detection of apple rubbery wood (ARW) (Waterworth, and Fridlund, 1989).

Other propagated apple varieties and rootstocks were infected with viruses or viruslike pathogens:

a) Jonagold cv. with ACLSV
b) P22 rootstock with ACLSV
c) P1 rootstock with ASPV
d) Golden Delicious with apple proliferation MLO and ACLSV
e) Lord Lambourne with ARW MLO and ACLSV

2.1.2. Micropropagation

Establishing of cultures

All source plants were potted and kept in cold room to pass the chilling period. Gradually, they were transferred to greenhouse, where they started to grow.

Explants were obtained from actively growing 2-3 year old plants. Shoot tips about 1 cm long, were cut off, sterilized and then shortened to 0.2-0.3 cm. The cultures were established on Zhao medium (Zhao, Hui-Xiang, 1982), containing half of macroelements and one tenth of microelements of Murashige and Skoog (1962) to which BAP 0.5 mg/l was added.

Shoot multiplication

Multiplication of shoots was carried out mainly on MS medium with 0.75-4.0 mg/l BAP, 0.05 mg/l NAA, 1 mg/l thiamine, 0.5 mg/l piridoxine, 0.5mg/l nicotinic acid, 2 mg/l glycine, 30 g/l sucrose and 7 g/l Difco Bacto-agar.
In the following experiments, to improve the proliferation of cultures, the media with different levels of BAP and auxins were tested. Proliferating cultures were kept in growth chamber at 22°C with 16 hours photoperiod by 1500 lux of cool white fluorescent lamps. The behaviour of cultures and proliferation rate were checked after four weeks.

Rooting of shoots.

After 6 months of stabilized growth 2-3 cm long shoots were transferred to Woody Plant Medium (WPM) (McCown, B.H., G. Lloyd, 1981) supplemented with 30 g/l sucrose, 0.5-1.5 mg/l IBA, 200 mg/l arginine, 0.5 mg/l Vit B2 and 6 g/l Japanese technical agar. The cultures were placed in growth chambers with no light for first 5 days and temperature 25°C. Later, the cultures were kept in 25°C and 16 h photoperiod.

2.1.3 Micrografting

The micrografting was made as soon as suitable shoots of indicator plants and plants used as a source of virus were obtained. At first the single shoots

from proliferating cultures were taken for micrografts. Later on single shoots with virus source were grafted to the whole proliferating culture.

Four indicators were used: Russian Seedling - for ACLSV virus, Virginia Crab - for stem pitting virus, Golden Delicious - for apple proliferation MLO and Spy 227 for Spy epinasty and decline, and ACLSV.

For successful micrografting it was essential to have thick shoots, with about 2 mm diameter. Such shoots were usually available in cultures of Russian Seedling
R 12740-7A and Virginia Crab indicators, P22 rootstocks and Jonagold cv. Golden Delicious and Spy 227 produced very thin shoots. To improve their quality paclobutrazol at concentration of 0.2-0.5 ppm was added to the medium.
The single micrografted plants were placed into tubes or flasks. Also, to some media activated charcoal was added.

The following combinations were used in micrografting:

Russian Seedling indicator and P22 infected with ACLSV
Russian Seedling " and P22 healthy rootstock (control)
Russian Seedling " and Golden Delicious with apple proliferation MLO
Russian Seedling " and Jonagold with ACLSV
Spy 227 indicator and P22 with ACLSV
Spy 227 " and Jonagold with ACLSV
Spy 227 " and P1 with ASPV
Spy 227 " and P22 healthy rootstock (control)
Virginia Crab indicator and P1 rootstock with ASPV
Virginia Crab " and P1 rootstock healthy (control)
Virginia Crab " and Jonagold with ACLSV

The micrografted plants were observed every few days. Altogether about 400 grafts were made.

To stimulate the virus symptoms occurrence the cultures were kept in chambers where day temperature was set on 24ºC and night temp. on 20ºC in one chamber and on 27ºC and 16ºC respectively in other chamber. The plants were checked for ACLSV after 10 day treatment (Fridlund, 1970).

No significant differences in growth or symptoms expression were found in plants from both chambers. The experiment was repeated four times.

ELISA test for virus transmission and concentration

The only pathogen detected and measured in the conducted experiments was apple chlorotic leaf spot virus. The virus transmission in micrografted components was studied about three weeks after micrografting. Scion and rootstock parts of plants were tested for ACLSV. Two different antisera against ACLSV were used in most tests but no significant differences were noticed.

2.2. Results.

2.2.1. Establishing and proliferation of cultures

The Zhao Medium, as described above, appeared to be suitable for stabilization of all but two indicators and apple cultivars used in the experiments. However, the behaviour of various plants differed.

The most suitable for multiplication and growth was MS medium (1962) modified by doubling the dose of $MgSO_4$ with addition of 0.5-4.0 mg/l BA, 0.02-0.05 mg/l NAA, vitamins: 1 mg/l thiamine, 0.5 mg/l nicotinic acid, 2 mg/l glycine and 30 g/l sucrose, stabilized by 6 g/l Difco-Bacto Agar.

Russian Seedling R 12740-7A was the easiest to get established in the cultures. In case of Golden Delicious the higher (4 mg/l) dose of BAP was necessary to get good proliferating cultures.

Finally, we succeed to establish Spy 227, Jonagold cv and P1 rootstock.

Although the changes in medium were made we did not succeed in establishing Lord Lambourne and Malus platycarpa indicators.

2.2.2. The behaviour of obtained cultures

Russian Seedling R 12740-7A. The cultures of Russian Seedling were very vigorous and well proliferating at the beginning. Usually, 3-5 additional shoots were obtained from each plant. However, it was found that after 3-5 passages the plants lost their juvenility. To prevent this phenomenon, the cultures had to be kept in low temperature (0-4¬C) to pass the chilling period.

Virginia Crab - appeared to be more difficult for stabilizing in vitro. During first few passages the side shoots did not elongate but produced rosettes. Addition of double dose of $MgSO_4$ stimulated the growth. Higher dose of BAP increased the number of side shoots but the dose above 1 mg/l caused

vitrification of shoots.

Golden Delicious - only about 30% of explants survived the stabilization stage. The obtained plants grew quickly but their shoots were very thin. The addition af paclobutrazol to the medium caused deformation of shoots. Well proliferating cultures were obtained when higher
(4 mg/l) dose of BAP was added to the medium.

P22 healthy, P22 with ACLSV and P1 with ASPV, stabilized and proliferated fairly easily and produced good shoots.

The average proliferation coefficient amounted
2.5-5. The highest number of shoots produced Jonagold and Spy 227 cultures.

Rooting of cultures

The shoots were suitable for rooting after 5-6 passages which is in agreement with the results of Sriskandarajah et al (1982) and Orlikowska and Machnik (1984). The highest percent of rooted shoots was obtained with Virginia Crab, Spy 227 and Jonagold. In case of those species up to 90% of shoots placed on WPM with
0.5 mg/l IBA rooted. The shoots of Russian Seedling were not suitable for rooting, after losing their juvenility.

Only about 30% of Golden Delicious plants formed
the roots.

The rooted shoots were planted in pots filled with substrate, containing mixture of brown coal, with
sphagnum peat 2:1.

The plants grew quite well and are being used for testing in greenhouse.

2.2.3. Micrografting

The number of micrografted plants depended on
the numbers of plant material available. The addition of paclobutrazol did not improve the quality of Golden Delicious and Spy 227 shoots.

It was found that presence of virus influenced the amount of grafts taken and the behaviour of micrografted plants. It was especially evident in micrografting of ACLSV infected P22 rootstocks with Russian Seedling R 12740-7A

indicator. Similar reaction was observed when other cultivars like Jonagold or Golden Delicious infected with ACLSV were grafted with Russian Seedling indicator (Fot. 1,2).

The dieback of Russian Seedling was common phenomenon in grafts where this indicator was used as scion. In some cases also the virus introduced with infected scions of Jonagold or Golden Delicious caused death of Russian Seedling (Fot. 3,4) used as rootstock.

The response of Spy 227 indicator to grafts containing ACLSV was similar to reaction of Russian Seedling. Also in such combination the death of Spy 227 scions grafted to the rootstock with virus was a common phenomenon (Fot. 5). Very thin shoots obtained in cultures of Spy 227 made the grafting more difficult and less successful.

On some micrografted Spy plants symptoms similar to epinasty were observed although it was not as common as dying of single Spy shoots.

No reaction was observed on Virginia Crab on which the rootstock P1 with stem pitting virus was grafted. However, the grafts in this combination were made in low number and therefore no final conclusion can be drown. The control plants of Virginia Crab, on which healthy scions were grafted grew very well but sometimes

the browning of tissue in grafting place occurred (Fot. 6).

Golden Delicious infected with ACLSV together with apple proliferation pathogens caused stronger reaction on Russian Seedling indicator than Golden Delicious with ACLSV alone. In grafts of Golden Delicious with both pathogens the scions of all Russian Seedling were killed.

The addition of charcoal to the media caused better growth of micrografted plants. They had bigger, dark green leaves but no symptoms of ACLSV infection was observed on indicators.

In general, the control micrografts, in which both components were free of viruses grew very well and almost 100% of grafts were taken (Fot. 7,8).

2.2.4. Relation of ACLSV concentration measured in ELISA to the behaviour of grafted indicator plants.

Transmission of virus to Russian Seedling was quicker and reached much higher concentration, when the virus source was supplied as scions (Table 1). This was also true in case of Spy 227 indicator, although in that case the concentration of virus was not as high as in Russian Seedling.

Table 1. Transmission of ACLSV from infected P22 plants micrografted to Russian Seedling R 12740-7A indicator (average A_{405} values in four Experiments)

No of exp.	Date of test	Rootstock P22 with ACLSV	Scion R.Seedling ind.	Rootstock R.Seedling ind.	Scion P22 with ACLSV
1.	18.11.92	0.429	0.161	0.534	0.238
2.	20.04.93	1.050	0.380	3.980	1.660
3.	27.05.93	1.630	0.435	1.820	0.595
4.	27.07.94	0.896	0.196	0.423	0.255

2.3. Conclusions

The carried out experiments indicated that it is not easy to obtain apple rootstocks, cultivars and indicators as cultures in vitro. Almost each cultivar or rootstock type requires individual media with different doses of growth substances and diversified treatments.

We succeeded in producing cultures of the following apple plants:

1/ Indicators: Russian Seedling R 12740-7A, Spy 227 Virginia Crab.

2/ Cultivars: Golden Delicious (also used as indicator), Jonagold.

3/ Rootstocks: P22, P1, P60.

We did not establish cultures of indicators Lord Lambourne (used for detection of apple rubbery wood disease) and Malus platycarpa.

We had obtained rooted plants of indicators: Russian Seedling R 12740-7A, Virginia Crab, Spy 227, and Golden Delicious.

In general, the presence of virus in one component decreased the graft take of micrografted plants. No typical symptoms of infection were observed on any

micrografted indicators. The only reaction was dieback of Russian Seedling R 12740-7A, grafted on rootstock infected with ACLSV. Sometimes we observed also death of this indicator, used as rootstock. However that phenomenon was not observed on all micrografted plants. Usually the dieback of indicator was connected with high concentration of ACLSV - as it was found in ELISA tests.

No reaction was observed on Virginia Crab indicator grafted with P1 rootstocks obtained from plants with stem pitting virus. However, in this case the presence of virus in grafted plants was not checked, because ELISA is not available for this virus yet. This is also true for Spy 227 epinasty and decline virus.

The suitability of obtained in vitro indicators for detection of viruses and viruslike pathogens will be studied in greenhouse conditions. To have always available uniform indicators, especially detecting viruses undetectable by ELISA would be of great meaning for quarantine purposes. Therefore we will concentrate on checking the reaction especially of Virginia Crab and Spy 227 to viruses such as apple stem grooving, apple stem pitting and Spy epinasty and decline. Those are viruses latent in commercial cultivars and still highly frequent.

Obtained cultures of Jonagold and Golden Delicious infected with ACLSV will be used for experiments with heat therapy carried on in vitro grown plants.

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Fot. 1. Dying Russian Seedling indicator micrografted to Golden Delicious rootstock infected with ACLSV.

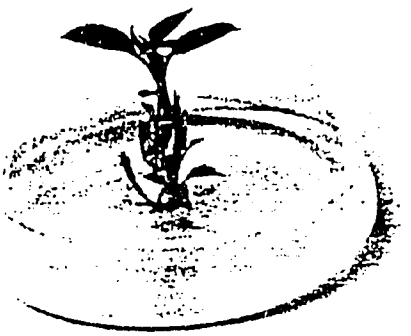
Fot. 2. Dying Russian Seedling indicator micrografted to Jonagold rootstock infected with ACLSV.

Fot. 3. Dying Russian Seedling indicato used as rootstock on which Jonagold with ACLSV was micrografted.

Fot. 4. Dying Russian Seedling indicato: used as rootstock on which Golden Delicious with ACLSV and apple proliferation was micrografted.

Fot. 5. Dying Spy 227 indicator shoots to which scion of ACLSV infected Jonagold was micrografted.

Fot. 6. Surface browning of healthy micrografted plant of Virginia Crab used as rootstock and P22 used as scion.



Fot. 7. Micrografted healthy control plant /scion and rootstock of Russian Seedling virus free/.

Fot. 8. Micrografted healthy control Virginia Crab indicator.

GENERAL CONCLUSION

The overall contribution of the present project to developing the *in vitro* indexing could be summarized according the progress made through the secondary objectives of the project.

The first objective was to develop tissue cultures of peach and apple cultivars that could be used as virus sources. This objective was basically achieved. Conditions for the entire stages of micropropagation systems, from tissue establishment until plant regeneration, have been developed for peach (Israel) and apple (Poland) systems.

The varieties that have been developed for use as PNRSV sources:

- *Prunus persica* L. (Peach) cv. Hermosa
- *P. persica* cv. Summerset
- *P. persica* cv. Babcock
- *P. avium* L. (Sweet cherry) cv. 40 E 50
- *P. avium* cv. 106 EB 325
- *P. amygdalus* Batch (almond) cv. Hazanov

For apple (Malus) system:

- Jonagold with ACLSV
- P22, P1 and P60 rootstocks with ACLSV
- Golden Delicious with apple proliferation MLO and ACLSV

More or less, a common basal medium have been adapted for peach cultivars.

The media were calibrated to each cultivar mainly through modification of hormonal compositions. Such common media may ease the introduction into tissue culture those *Prunus* varieties that have to be checked for their virus infection status through the *in vitro* indexing system.

For the apple system it was difficult to obtain such a common medium.

. Virus free and virus infected clones have been developed for all the tested cultivars. For some of the cultivars such as *P. amygadlus cv. Hazanov,*

conditions for virus elimination through *in vitro* thermotherapy and shoot-tip culturing had to be newly applied.

The second objective was to develop *in vitro* cultures of specific plant inhibitors to peach and apple virus diseases. This objective was also achieved. *P. persica* cv. Elberta, *P. serrulata* cv. Shirofugen and *P. tomentosa* have developed for the biological detection of PNRSV.

Russian Seedling R12740-7A, Virginia Crab and Spy 227 have been developed for detection of the apple virus diseases.

The third objective was to develop micrografting methods enabling virus transmission to indicators and symptom induction.

Clone selection, manipulation of media composition and addition of plant growth regulators had to be carried out for obtaining successful micrografting in a reasonable rates. Virus transmission to almost all indicators have been detected by ELISA. However, dieback symptoms have been observed only in Shirofugen indicator for PNRSV and Russian Seedling R12740-7A for ACLSV.

Many experiments have been conducted aiming for induction symptoms on the other indicators, but so far, with no significant success.

Applied results and proposed project continuation

Methods developed in the present project for micropropagation, micrografting, virus transmission and symptom induction enabled production and using the successful indicators for virus detection a whole year around, under controlled conditions. The proper conditions for symptom induction onto other indicators still have to be developed.

In vitro plant material had been already exchanged between the cooperating countries for introducing them into the practical procedures of the fruit tree quarantine system.