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Production of Virus-free Sweet Potato Planting Material for the Southeast of Kazakhstan

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Abstract

Sweet potato is culture of food security in the rural economy of many countries, as it is a highly process ability and easily cultivated, also sweet potato has the status of a dietary product. In this research article, our main goal was to develop the first in Kazakhstan biotechnology to obtain virus-free planting material of sweet potato from the introduction of apical meristem *in vitro* to produce virus-free planting material. In culture of apical meristem induced callus with subsequent regeneration of plants and for cloning *in vitro*, nutrient solution was optimized. As a result of PCR analysis, all obtained plants were free from viruses. Tubing plants were planted in the soil for propagation and subsequent production of tubers. Obtained results showed the possibility of growing virus-free planting material for further large-scale production of sweet potato in Kazakhstan. © 2018 Friends Science Publishers

Key words: Sweet potato; Apical meristem; RNA; Virus-free
Abbreviations: SPFMV, Sweet potato feathery mottle virus; SPCSV, Sweet potato chlorotic stunt virus; SPVD, Sweet potato virus disease; MS, Murashige and Skoog; BAP, 6-benzylaminopurine; dNTP, deoxynucleoside triphosphate; M-MLV, Moloney Murine Leukemia Virus

Introduction

Sweet potato can be a good addition to the dietary deficiency in Kazakhstan. Since sweet potato is not traditional culture in the Kazakhstan, for industrial production it is necessary to create cultivation technology of high-quality planting material. Sweet potato *(Ipomoea batatas)* – a tuber plant, belongs to the *Convolvulaceae* family, and *Ipomoea* genus, valuable food crop and fodder culture. Sweet potato is a grassy liana with long 1−5 m creeping lianas rooted in the nodes. The height of the bush is 15−18 cm. The lateral roots of sweet potato thicken strongly and form tubers with white, yellow, orange, pink, cream, red or violet edible pulp. One tuber weighs from 200 grams to 3 or more kilograms (Sunette *et al.*, 2015).

Sweet potato is cultivated in tropical and subtropical regions of the globe, sometimes in warm regions of the temperate zone. It is especially widely grown in China, India and Indonesia. Worldwide, sweet potato is the sixth most important food crop after rice, wheat, potato, corn. More than 106 million tons of sweet potato is produced annually, furthermore developing countries produce 95% of total production (Williams *et al*., 2013). Due to the simplicity of cultivation and high technology, sweet potato is considered a food safety culture and a staple food in the rural economy of many countries (Yamakawa, 1998). Importance of sweet potato as a food crop is growing rapidly in some parts of the world: Southeast Asia, sub-Saharan Africa, South America.

Tubers of sweet potato up to 30 cm long, juicy, with a tender pulp and thin skin peel. They do not have “eyes”, and sprouts develop from hidden buds. Tubers of different varieties can vary greatly in form - round, oval, elliptical; by the color of the pulp - white, yellow, orange, cream, violet; by the taste - from insipid to very sweet; by the texture - from soft and juicy to dry and hard; by the color of the peel - almost all the colors of the rainbow. Most of the cultivated varieties are more or less sweet, because of sucrose, glucose and fructose. Milk juice appears on the rupture of the tuber (Zhang *et al*., 2009).

The composition of tubers can vary depending on the particular cultivar and growing conditions (climate, agrotechnology). Sweet potato with orange pulp is an important source of beta-carotene, and provitamins of A group. For example, in 125 grams of fresh tubers of sweet potato of most varieties with orange flesh, are enough beta carotenes to provide a daily requirement with pro-vitamin A of the preschool child. Sweet potato is also a valuable source of vitamins B6, B2, C, E and contains a sufficient amount of copper, manganese, iron and zinc. Nutritionists in the United States are studying the potential prevention of cancer with the properties of sweet potato with violet pulp

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(Hill *et al*., 1992).

Anthocyanins, which form violet pigmentation in tubers (also in berries and vegetables such as blueberries and red cabbage) are powerful antioxidants and have good bioavailability, which means that they are easily absorbed from the gastrointestinal tract into the bloodstream. In addition, sweet potato has the status of a dietary product, applied as a vitamin and fortifying agent (Yudi *et al*., 2015).

Despite the name "sweet", sweet potato can be used as a dietary product, it helps to stabilize blood sugar levels and reduce insulin resistance. Sweet potato propagates vegetatively and therefore resistance to viral diseases for planting material is an important condition for production. Yield losses caused by these viral disease range from 20% to 40%. More than 30 viruses can infect sweet potato. Sweet potato feathery mottle virus (SPFMV) is the most widespread virus infecting sweet potato. The synergistic interaction of SPFMV with Sweet potato chlorotic stunt virus (SPCSV) causes the development of Sweet potato virus disease (SPVD). The yield reduction reported for this disease is reported to be 65 to 72% on different cultivars, depending on the cultivar. To produce the virus-free sweet potato material, *in vitro* culture of meristems can be used. For sweet potato meristem isolation, upper apical meristems should be taken this group are free from viruses, since the division of cells in this part of the plant is most intense (Henderson *et al*., 1984).

At the same time, we should not forget about explant totipotency of the implant depending on the taken size of the meristems, since the genotype affects on growth and development of the plant. In addition, the use of apical shoots for the isolation of meristems is considered better than the use of shoots from the side kidneys. The first who reported about the use of meristems for the production of virus-free sweet potato was Nielsen (1960).

Subsequently, Mori (1971) showed the removal of important virus of sweet potato by using meristems (SPFMV). Afterwards, Alconero *et al*. (1975) placed apical shoots of ten varieties 0.4−0.8 mm in size, on a nutrient medium MS, with the addition of auxin and cytokinin. As a result, 47% of the vaccinated plants were free from viruses. Axenic meristem culture 0.25−0.4 mm in size was used, in the work of Frison and Ng (1981) to obtain virus-free sweet potato plants, however result was negative. In the work of Liao and Chung, were unable to obtain sweet potato virus-free material by thermotherapy 38−42°C, for 30−90 days cultivation of a 50 mm top shoots. Also it had not been possible to eliminate viruses from meristems with a length of 5 mm. As a result of the study, it was found, that the virus-free material can be obtained by culturing meristems with a length of 0.3-0.6 mm, taken from plants after thermotherapy 38−42°C for 4 weeks (Liao and Chung, 1979). The preparation of virus-free sweet potato discussed by Love *et al*. (1987) they describe procedures of meristem isolating, plants regenerating and testing for the presence of the virus. They emphasize the necessity to use a small explant size (0.1 mm) that contains an apical shoot meristem without the primordia leaf rudiments that were removed during the dissection.

Obtaining the virus-free material of sweet potato by apical meristem culture was reported many times and it should be noted that the isolation protocol varies insignificantly, however the nutrient media for cultivation, as well as the conditions for cultivation of meristems change, depending on the sweet potato cultivar. Purification of sweet potato from viruses by meristem culture is considered to be much more effective than thermotherapy, with 80% obtaining virus-free plants. Nevertheless, there are reports of the receipt of virus-free planting material of sweet potato, with thermotherapy (Huett, 1982).

On the basis of the literature, most important ways to control sweet potato viral diseases is to obtain healthy seed material and accelerated reproduction, based on a tissue culture method. This indicator is now mandatory in the production of sweet potato, because it affects on yield quality and quantity. Moreover, it is known that virus-free planting material is more sustainable to other pathogens (fungus, bacterial) and abiotic stress factors. In connection with this, developed countries the seed planting material of vegetatively propagated crops must be virus-free. In Kazakhstan there is no industrial production of sweet potato. At the same time, there is a requirement in new cultures for healthy and dietary nutrition. For large-scale industrial production in Kazakhstan, indispensable condition will be the availability on the market of high-quality virus-free sweet potato seed material.

Materials and Methods

Materials

20 lines of sweet potato are received from department of System of Engineering of Plants, the Korean Institute of Biology and Biotechnology.

Methods

Planting sweet potato tubers under controlled conditions, obtaining lianas: Tubers planted in ground under controlled conditions horizontally, to obtain a larger number of sprouted lianas from hidden buds, temperature was kept at 23−26°C with a light mode of 16/8 (day / night), 10,000 lux, humidity 50−60%. Irrigations were applied twice a week. Lianas were grown to 0.8−1 m, for subsequent propagation.

Method of isolating the apical meristem of sweet potato: The tops of shoots from actively growing lianas were cut off, washed under running water for 30 min, and sterilized with a 2.5% solution of sodium hypochlorite for 5−10 min, then in 70% alcohol for 3−5 sec, inside the laminar box. Treated explants then washed in sterile distilled water. Apical meristems shoots were isolated using a sterile needle

and scalpel, under a binocular microscope MBS-10 in a laminar box. Isolated apical meristems (0.3-0.5 mm) were transplanted on a MS nutrient medium (Murashige and Skoog, 1962) with a kinetin 2.5 mg/L and gibberellic acid 0.5 mg/L hormone (Alam *et al*., 2004; Alam *et al*., 2010).

Propagation method of sweet potato liana: Cuttings of sweet potato were carried out according to the following protocol: lianas of the appropriate size were divided on cuttings 7−8 internodes, while the lower 2−3 leaves were removed. Cuttings were then placed in a root solution (2 g/L of water) to obtain rootlets and then planted. Cuttings were planted at an angle of 45°, 3−4 interstices were buried in the ground. Between cuttings left a distance 30−40 cm, between lines 100 cm, distance between rows was 80 cm. The first 2−3 weeks of lianas grew slowly at the 4th week active growth began. Weeding was done 2 times a week.

Nutrient medium optimization for cloning regenerants: For optimization of nutrient media optimization for cloning regenerants, two different nutrient media were used, MS with 1% sucrose and 0.05 mg/L BAP hormone and MS without hormones but with half salt concentration and 3% sucrose. Cloned sweet potato plants were grown to a five leaves phase and transplanted from the test tubes to the ground under controlled conditions. An important stage in transplantation maintaining high humidity during transplantation to the ground was maintained due to a plastic cup installed on top, also by a temperature 25°C and 10000 lux illumination, with a light mode 16/8 (day/night). Plastic cups with regenerants were placed on a pallet and poured from the bottom, in order to provide air to the root system. For plant adaptation, it was important to maintain a high relative humidity (60−80%) during the first week.

After transplanting in soil the plants were fertilized with "Kemira Hydro" (1 g/L of water) containing magnesium, sulfur, and microelements. The watering was carried out once in 2 weeks. To stimulate growth, "Kornevin" solution (1 g/L of water) was also applied once. Plastic cups after 10 days slightly opened, for plant adaptation to the greenhouse conditions. Watering was carried out every 2 days. After three weeks all lines of sweet potato showed intensive growth of lianas. After 4−5 weeks, the lianas reached a length of 0.8−1 m.

RNA isolation: For viral diseases detection, purification of RNA using Trizol (Molecular Research Center Inc.) method, with modifications, was used (Chomczynski and Sacchi, 1987). The tissue sample was homogenized in 1 mL of Trizol, incubated on a thermoscheaker for 5 min at 65°C, centrifuged for 10 min at 14,000 rpm, then incubated on a thermoshaker at 65°C for 10 min. 200 µL of chloroform was added, gradually mixed for 5 min, after which it was centrifuged for 10 min at 14,000 rpm and the aqueous phase was transferred to a new tube, an equal volume of isopropanol was added and mixed. Then content were placed in a freezer for 30 min at -20°C, centrifuged for 5 min at 14,000 rpm, and the precipitate was washed with 70% ethanol, dissolved in deionized water and stored at -70°C.

The procedure of reverse transcription was carried out using the Sileks equipment (Russia) in accordance with the protocol and the manufacturer's recommendations. Mix No. 1 volume 18 µL containing 2 µL of total RNA (0.1−5 µg), 1 µL of specific primers (15−20 pmol), and RNase free water. The mixture was incubated for 5 min at 70°C. Then, mixture No. 2 was prepared, 7 µL in volume, containing 2.5 µL of 10 × reverse transcription buffer, 4 µL of a 2.5 mM mixture of each of the four dNTPs, 0.5 µL of M-MLV Sileks reverse transcriptase (200 units/µL). Next step, mixtures No. 1 (18 µL) and No. 2 (7 µL) were combined and incubated for 60 min at 37°C, then reaction was stopped by heating the mixture for 10 min at 70°C.

Amplification of DNA by Polymerase Chain Reaction (PCR)

For PCR analysis, a 20 µL reaction mixture was prepared having the following composition: 2 µL of 10x HotTaq polymerase buffer, 2 µL 2.5 mM dNTP mix, 1 µL primer mixture 50 pmol concentration, 0.5 µL of HotTaq polymerase, 2−5 µL DNA, 8−11 µL of RNA free water (Table 1).

Temperature reaction regime:
Step 1 - 94°C 15 min. - 1 cycle
Stage 2 - 94°C 1 min, 58−60°C 1 min, 72°C 1 min. - 33 cycles
Stage 3 - 72°C 5 min. - 1 cycle

Results

In our previous studies, we conducted research on characterization and formation of a working collection of sweet potato according to the standards of the International Potato Center (CIP). 20 genotypes, received from the Korean Institute of Biology and Biotechnology, were analyzed and described (Zatybekov *et al*., 2015).

Afterwards, 3 years of testing in the southeast of Kazakhstan from 20 sweet potato lines selected 6 lines, with the highest yield, an average tuber size and good taste qualities - K7, K12, K13, K15, K20, and K8 line having bright orange color, which indicates a high content of beta-carotene. The tubers of the above-mentioned lines were planted in the soil under controlled conditions to obtain lianas and subsequent isolation of the apical meristems.

When lianas reached the appropriate size, apical meristems of sweet potato were isolated, and transferred to the nutrient medium of MS with hormones: kinetin - 2.5 mg/L and gibberellic acid - 0.5 mg/L. During the cultivation of apical meristems, callus was formated. Afterwards, on the same medium, 2 months later, the plants formed from calli (Fig. 1).

Table 2 presents the results of the obtained regenerants from the apical meristems. All studied lines

Table 1: Primers for RT-PCR analysis for detection of sweet potato viruses

Name	Oligonucleotide sequence of primers	Fragment size (b.p)	Annealing temperature (t^0)
SPFMV	F. TACACACTGCTAAAACTAGG	356	58
	R. AGTTCATCATAACCCCATGA		
SPLV	F. GGAGTCAGTTCAATCAATGGTA	184	60
	R. AGTGGCTTTATTGGGTATGAT		

Table 2: Regeneration in culture of apical meristems of sweet potato

Line name	Callusogenesis, %	Plants regeneration from calli, %
K7	40	60
K8	80	20
K12	55,5	44,4
K13	60	40
K15	80	20
K20	80	20

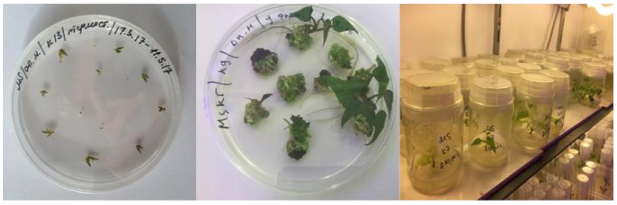
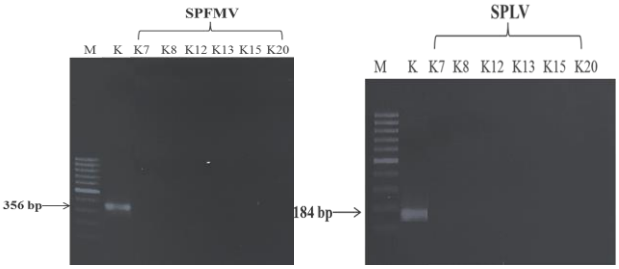


Fig. 1: Development of isolated apical meristems on a nutrient medium



M - marker; K - positive control

Fig. 2: Electrophoresis for the presence of SPFMV and SPLV viruses

formed callus with different frequency.

The process of regeneration of plants from calli continues considerable time from two to four months. The number of regenerants was from 20 to 60% of the number of cultivated calli, depending on the genotypic characteristics of the studied sweet potato lines.

To clone obtained regenerants, nutrient medium was further optimized. For nutrient media optimization, two MS mediums containing 1% sucrose, and 0.05 mg/L BAP hormone and MS without hormonal with half salt concentration and 3% sucrose were used (Table 3).

As a result of the research, it was found that cultivated on a nutrient medium MS with 3% sucrose, half content of salts and without hormones, germination of the axillary bud and root formation was much faster, and

a b

Fig. 3: a - sweet potato the feathery mottle virus, b - sweet potato latent virus

Fig. 4: Cloned plants development from the apical meristems under controlled conditions

also plants reached the phase of the fifth leaf faster. Whereas on MS medium content 1% sucrose and 0.05 mg/L BAP hormone, wilting and discoloration of the leaflets were observed. However, all tested lines showed good root formation in both media.

Then, from the apical meristem obtained from cloned plants, total RNA was isolated using Trizol, followed by reverse transcription and cDNA isolation. A PCR analysis showed presence of two main viruses, SPFMV and SPLV (Fig. 2).

The result showed absence of SPFMV (sweet potato feather spot virus) and SPLV (sweet potato latent virus) viruses in regenerants obtained from apical meristems. Fig. 3 shows the external appearance of the infected plants with the above mentioned viruses, which were used as a control.

Cloned sweet potato plants were germinated to the phase of five leaves and transplanted from the test tubes into the soil under controlled conditions. After 4−5 weeks, the lianas reached a length of 0.8−1 m. Growth rate and quality of lianas practically did not differ from lianas obtained from tubers (Fig. 4).

After reaching the appropriate size lianas, were divided into cuttings, 7−8 internodes, lower 2−3 leaves were removed. Cuttings were planted in open and closed ground. To optimize the adaptation of sweet potato in the ground, 3 types of substrate were used: mixture 1: sand, soil and organic fertilizers 2:2:1; mixture 2: sand, soil and organic fertilizers 1:2:2; mixture 3: sand, soil and organic fertilizers 2:1:2.

Table 3: Clone development on nutrient media (in days), from the day of planting regenerants on a nutrient medium

Liana	Root appearance			Full root			Axillary buds germination			Fifth leaf appearance		
	MS +3% sucrose	w/h*	MS+BAP 0.05 mg/L+1% sucrose	MS +3% sucrose	w/h*	MS+ BAP 0.05 mg/L +1% sucrose	MS +3% sucrose	w/h*	MS+ BAP 0.05 mg/L+1% sucrose	MS +3% sucrose	w/h*	MS+ BAP 0.05 mg/L+1% sucrose
K7	4		10	15		27	7		12	22		34
K8	5		12	15		30	7		16	24		34
K12	4		14	18		25	6		12	27		36
K13	3		11	15		25	9		13	23		34
K15	4		11	16		29	7		11	23		37
K20	4		12	15		30	9		10	27		34

* without hormones

Phenological observations showed that active growth of lianas begins after 3 weeks of planting. The soil for cultivation of sweet potato should be light, optimal mixture was 1 (sand, soil, organic fertilizers 1:2:2) and mixture 3 (sand, soil, organic fertilizers 2:1:2), plants on mixture 3 were more bushy, possibly due to more organic fertilizers. During the growing season, flowering was observed in the two lines K7 and K13. Tubers formation began after two months planting in the ground.

Discussion

Sweet potato a vegetatively propagated culture, therefore it has the properties of being exposed to viral infections through vines and tubers, which leads to serious diseases. Research has been carried out using different approaches including biological and molecular assays for obtaining a virus-free material and detecting known viruses (Abad and Moyer, 1992; Colinet *et al*., 1993, 1998; Chavi *et al*.,1997; Mukasa *et al*., 2003, 2006; Souto *et al*., 2003; Ateka *et al*., 2004; Kokkinos and Clark, 2006; Zhang *et al*., 2010). Sweet potato has a very high genetic variability. Consequently, the differences in the response of tissue culture to the nutrient medium can be quite strong, depending on the genotype. To obtain plants from isolated meristems, it is usually necessary to add hormones to the nutrient medium.

In a present study, to obtain a virus-free planting material of sweet potato, apical meristems (0.3−0.5 mm) were isolated with the use of MS nutrient medium and the contenting hormones kinetin and gibberellic acid, as previously studied and shown in the work of Love *et al*. (1987). Selected apical meristems of 6 lines were cultivated on a nutrient medium with hormones, calli formed with different frequencies. The process of callus regeneration lasted from two to four months. As a result, the number of regenerants obtained varied from 20 to 60%, depending on the genotypic characteristics of the studied sweet potato lines.

It is highly important to use carbohydrates to promote plant growth and subsequent regeneration. For sweet potatoes, the best carbohydrate is sucrose, used as a transport form of carbon and energy. However, high sucrose content of more than 5% adversely affects the development of sweet potatoes (Thompson and Thorpe, 1987). The role of osmotic pressure of sucrose on plants was also reported (Lipavsksa and Vreugdenhil, 1996).

In a current experiment, for regenerants growth two MS medium were tested: MS medium containing 1% sucrose with BAP hormone 0.05 mg/L and MS containing 3% sucrose, half salt concentration, without hormones. On MS medium with 3% sucrose, was observed accelerated germination of the axillary bud and root formation, and the development of the plant was more intensive. Whereas, MS medium with 1% sucrose adversely influenced the plants.

Obtained results are consistent with earlier scientific studies by Thompson and Thorpe (1987), where it was shown that high (5%) and low (1%) sucrose concentration, as well as in our studies, negatively affected the plant. Optimal percentage of sucrose for cultivation of sweet potato was 3%.

According to the results of RT-PCR analysis, all plants obtained from apical meristems were free from SPFMV and SPLV viruses. The researchers Rukarwa *et al*. (2010) and Xiansong (2010) collected identical results. This study also shows the significance of the apical meristems culture for obtaining virus-free plants of sweet potatoes. Previous studies show that the elimination of the virus, is due to the fact that viruses do not penetrate into the cells of the apical meristem, because of actively dividing tissues.

Conclusion

As a result of the experiments, a virus-free planting material of sweet potato was obtained. It has been demonstrated that virus-free material of sweet potato can be propagated in the South-East of Kazakhstan. The conducted research will serve as a basis for mass production of high-quality planting material in Kazakhstan.

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