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Utility of Thermotherapy and Meristem Tip for Freeing Sweetpotato from Viral Infection.

Mervat M. M. El Far and A. Ashoub

Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre (ARC), 9 Gamaa St. 12619, Giza, Egypt

Abstract: Sweetpotato, (*Ipomoea batatas L.*), is vegetatively propagated crop. Due to the following cycles of propagation, viruses are accumulated, which contributes to the decline of sweetpotato yield and quality. Stem cuttings of Abees cultivar were collected from several locations in Egypt and kept under controlled greenhouse conditions. Samples were tested for the presence of sweetpotato feathery mottle virus (SPFMV) infection using dot-ELISA. Infected plants were thermotherapy treated by incubation of plants at 42°C/ day and 39°C/ night for 3 weeks followed by meristem tip culturing and allowed to grow *in vitro*. RT-PCR was carried out to confirm the success of SPFMV elimination. Tissue culture formed plants were tested routinely for successive 2 years using dot-ELISA. 0% infection was reported in the *in vitro* propagated plants. Sweetpotato plants were assisted the private-sector to enhance the final yield in their bio-farming system.

Key words: sweetpotato; virus-free; dot-ELISA; thermotherapy.

INTRODUCTION

Sweetpotato (*Ipomoea batatas L.*) is a dicotyledonous, perennial plant, belongs to the family convolvulacea (Astin D.F., 1987). It ranks as the seventh important staple crop in the world and the fifth in developing countries after rice, wheat, maize and cassava (Loebenstein G., *et al.*, 2003). Sweetpotato considered being the second important root crop after cassava in many tropical countries (FAOSTAT, 2006). Because of the enormous genetic diversity of sweetpotato (Zhang D., *et al.*, 1998), and the accompanying diversity in phenotypic and morphological traits (Woolfe J.A., 1992), the crop has great potential for further development to accommodate specific uses. On the other hand, nutritional studies in Egypt gave the evidence that vitamin A deficiency (VAD) is prevalent among pre-school children and their mothers (Nutrition Institute, 1995). VAD is a main cause of child blindness, and even in its less acute forms, it hinders normal growth and development (WHO nutrition report, 2003). Orange fleshed sweetpotato varieties are rich in B-caroteen, the precursor of vitamin A, (CIP, 2001). In Egypt, the sweetpotato variety Abees, a purple skin and orange flesh, has a high productivity per unit/area, good nutritional value and a big demand for exportation. Abees cultivar represents approximately 60% of the total sweetpotato acreage (30.000 acres).

Despite of the advantages that the cultivation of sweetpotato offers, production is greatly constrained particularly by viral diseases (Tairo, F., *et al.*, 2005; valverde, R.A., *et al.*, 2007; Karyeija, R.F., *et al.*, 1998). Since Sweetpotato is cultivated from shoot cuttings grown in the field from previous season, percentage of virus infection increases, as consequence, total yield is dramatically reduced. The most common of these viral diseases known under the name Sweetpotato Virus Diseases (SPVD), caused by simultaneous infection with Sweetpotato Feathery Mottle Virus (SPFMV) and Sweetpotato Chlorotic Stunt Virus (SPCSV), (Winter, S., *et al.*, 1992; Gibson, R.W *et al.*, 1998).SPFMV (SPFMV; family potyviridae, genus potyvirus), is found most commonly in sweetpotatos in different parts of the world (Loebenstein G., *et al.*, 2003; Tairo, F., *et al.*, 2005). The potyvirus enhances accumulation of the unrelated virus (Karyeija, R.F., *et al.*, 2000; Latham, J.R. and A.K. Wilson, 2008). SPFMV, alone does not cause major yield losses, but co-infection and the consequent synergistic interaction of these two viruses cause the development of the sever SPVD, (Schaefers, G.A and E.R. Terry, 1976), which results in the reduction of sweetpotato yield for up to 90% while the infection of each virus alone results in reduction of up to 10% (Ishac, J., *et al.*, 2000; Carey, E.E *et al.*,1999; Gutierrez, D.L *et al.*, 2003; Njeru, R.W *et al.*, 2004; Mukasa, S.B *et al.*, 2006). Owing to the great yield losses caused

Corresponding Author: Mervat, M.M.EL FAR., Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre (ARC), 9 Gamaa St. 12619, Giza, Egypt
Email: ahmedmervat@yahoo.com

by virus diseases, their absence from propagation materials is essential for sustainable plant production. This is particularly important with vegetatively propagated crops in which infected planting materials transmit the pathogen to the following generation. Shoot tip, meristem tip and heattherapy have been widely used to produce virus-free clones of crop plants which are propagated vegetatively (Faccioli, G., and F. Marani, 1998). Sweetpotato was one of the first crops subjected to virus eradication with this technique (Alconero, R., *et al*., 1975; Frison, E.A. and S.Y. Ng, 1981; Green, S.K., and C.Y. Lo, 1989). The previous studies (Abo El-Abbas, F., *et al*., 1998) demonstrates the presence of SPVD in Egypt and its effect on sweetpotato production, and emphasized the need to control it by developing a system to produce virus-free plant material.

The aim of the present study is to improve the agronomic quality of deteriorated local cultivar, Abees using an efficient method for virus elimination by companying thermotherapy technique with meristem tip culture.

MATERIALS AND METHODS

Plant Material:

Sixty five sweetpotato Abees cultivar apical cuttings in a length of 15 cm were collected from several locations in Kafr El Shikh governorate, and kept under insect proof greenhouse.

Dot-ELISA:

Cuttings were tested for the presence of SPFMV using dot-ELISA following the method described by (Gutierrez, D.L *et al*., 2003) and the modifications of (Ashoub, A. *et al* 2008). Samples were tested using antiserum raised against SPFMV, kindly provided by International Potato Centre (CIP), and a polyclonal alkaline phosphatase labelled goat anti rabbit as secondary antibodies (Sigma, USA).

Thermotherapy:

Plants were subjected to the thermotherapy treatment as follows:

Plants were placed in a growth chamber under the conditions of 16 hours photoperiod at 28 ºC and 8 hours dark period at 25 ºC. Temperatures were raised 2 ºC/ 2 days till final setting of 42 ºC day and 39 ºC night was obtained. Plants were maintained under these conditions for additional 4 weeks.

Meristem Tip Culture:

Following thermotherapy, axillary shoot tips with a long of 2-3 cm were cut, surfaced sterilized by immersing in 90% ethanol for 10 seconds followed by 2% sodium hypochlorite solution, 0.05% tween-20 for 5 minutes and rinsing for three times, 2 minutes each, with a sterile distilled water. A part of the apex of approximately 0.25 mm long with two leaf premordia was excised for culture on a 5-cm Petri dish containing 15 ml of MS salts medium (Murashige, T., and F. Skoog, 1962) supplemented with 3 % sucrose. The cultures were grown at 28 ± 2 ºC under a 16-h photoperiod of 300 foot candle.

Virus Indexing:

Elongated shoots were tested for success of eradication of SPFMV using reverse transcriptase polymerase chain reaction, RT-PCR, as follows:

Total RNA was extracted from 50-100 mg sweetpotato leaves using SV total RNA extraction kit (Promega, USA) following the manufacturer recommendations. Reverse transcription and PCR reactions were carried out in according to the described method (Ashoub, A. *et al*., 2008).

SPFMV-free sweetpotato plants were massive *in vitro* propagated via nodal cuttings using the media and incubation conditions described above. To assure the absence of SPFMV from the micropropagated plant materials, leaf samples were collected every 2 months and tested by dot-ELISA as described above.

RESULTS AND DISCUSSIONS

SPVD which caused by SPFMV and SPCSV is one of the most important complexes, effort to develop virus-free cultivar to one of this viruses seemed sufficient to control SPVD.

Incidence of Sweetpotato Feathery Mottle Virus (SPFMV):

Serelogical detection methods such as most types of ELISA lack the sensitivity required for the detection of SPFMV in sweetpotato tissue. (Esbenshade, P.R. and J.W. Moyer, 1982). However dot-ELISA is more sensitive than microplate ELISA (Abad, J.A and J.W. Moyer, 1992; Gibbs, K.S and A.C. Padovan, 1993; Fuents, S. and LF. Salazar, 1992).

Dot-ELISA carried out on sweetpotato plants collected from Egyptian fields showed 100% infection (fig.1), in all, 65 collected sweetpotato samples. In Egypt, Isahck, J.A. *et al.,* 2003; and F. Abo El-Abbas *et al*., 1998) found that SPFMV is predominant in all planting material. Sweetpotato is propagated vegetatively using cuttings either produced as sprouts from tuberous roots or obtained from the foliage of mature crops (CIP, 2001), if when infected tuberous roots from a previous crop sprout in the same field or in areas adjacent to new crop, they serve as a source of virus inoculum (Karyeija, R.F., *et al*., 1998). The majority of the farmers (96%) obtain planting material from their stocks or borrow vines from neighbours. Due to lack of knowledge, most farmers (70%) select planting viens from healthy looking vigorously growing plants (Ateka, M.E. *et al*., 2004). However they did not recognize virus-like symptoms as a disease but associated viral symptoms with other factors such as drought, insect pests, and cultivar-specific behaviour. Lack of SPFMV symptoms as mono infection on sweetpotato plants prevents SPFMV-free plants of being selected as sources of cuttings (Clark, C.A. and J.W. Moyer, 1988; Brunt, A.A., *et al*., 1996; Gibson, R.W. *et al* 1997). This is probably explaining the wide distribution of the virus diseases. Absence of visual symptoms in sweetpotato is an inadequate test for SPFMV, since the virus cause slight or no symptoms when infected alone.

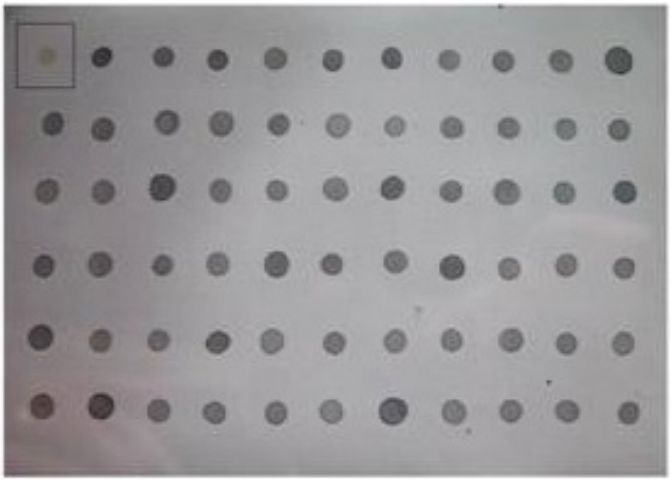

Fig. 1: Dot-ELISA of sweetpotato sample collected from egyptian fields. Healthy sample is indicated in a box at the top left handsite.

Effect of Heattherapy and Meristem Culture on Survival and Regrowth:

Nearly, all (100%) of the heattherapy treated plants survived. The gradual regime used for raising the temperature allowed plants to survive. However the regrowth ability of the meristem tip decreased, only 96% of the meristem tips survived and formed shoots, when meristem tips with two leaf premordia with the size of 0.25 mm were used as plant material (fig.2-A). These results are in agreement with the results of (Faccioli, G. and F. Marani, 1998; Fuglie, K.O. *et al*., 1999). They reported that meristem tips of 0.2 and 0.3 mm with two leaf primordial are commonly used for virus elimination in various plant species, and the survival and growth are greatly improved if the tissue taken includes slightly expanded leaf primordial.

Effect of Heattheraby Treatment and Meristem Tip Culture on Virus Elimination:

Out of sixty five SPFMV infected plants, sixty two SPFMV virus-free plants become established. (fig. 2-B). This study showed that SPFMV can be very efficiently eliminated from sweetpotato infected plants using the traditional heattheraby in a combination with meristem tip culture.(Cooper, V.C. and D.J.A. Walkey, 1978; Walkey, D.J.A. and V.C. Cooper, 1975; Hu, C.U. and P.J. Wang, 1983; Mink, J.I. *et al*.,1998;). While elimination of viruses by meristem culture is size dependent, it was recommended that the smaller the tip that is taken, the better chance for virus exclusion (data not shown), it comes with the findings of (Michael, J.K., 1996), because in the merastimatic zone the rate of plant growth is increasing comparing with the rate of virus multiplication. Meristem culture technique has regenerates over 35 genera (Quak, F., 1987). In contrast (Lankes, C.1995; Karesove, R., *et al*., 2002; Theiler-Hedtrich, R. and G. Baumann,1989) reported that meristem tip culture alone obtained a few virus-free plants. However thermotherapy followed by meristem culture resulted in SPFMV-free plants. This finding raised the question of the mechanism by which thermotherapy enhanced eradication of SPFMV. Early studies indicated that thermotherapy inhibits viral replication while virus

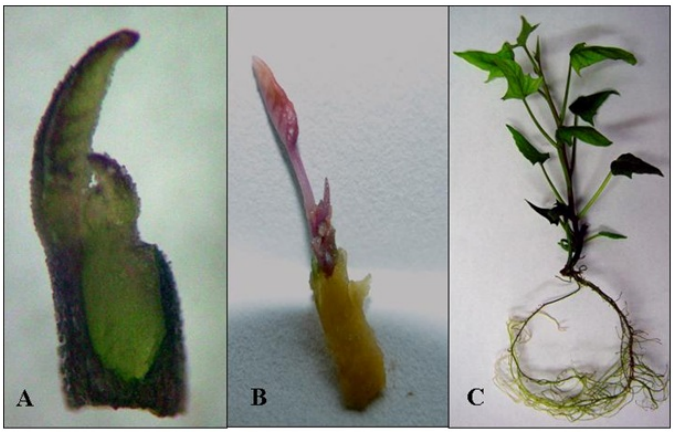

Fig. 2: Production of viru-free sweetpotato plants using heatherapy and meristem tip culture.
(A) Apical meristem tip (0.25mm with two leaf primordia) used for virus eridcation.
(B) Regrowth of meristem tip after 4 weeks of culture on MS medium supplemented with 3% sucrose.
(C) An established healthy plant of sweetpotato Abees cultivar obtained form SPFMV-affected plant using heattherapy and meristem tip culture.

degradation continues, which results in subsequent elimination of the virus from meristem tips (Cooper, V.C. and D.J.A. Walkey, 1978; Kassanis, B., 1975), later (Szittya, G., *et al*., 2003; Qu, F., *et al*., 2005; Chellappan, P., *et al*., 2005) found that, the efficiency of virus induced RNA silencing found to be significantly enhanced at the high temperature. Indeed, since, the first studies that aimed to produce virus-free plants from infected individuals, it has been known that the efficiency of virus eradication in a given host species differs depending on the virus and the host genotype. With some plants, thermotherapy followed by meristem tip culture did not result in any virus free plantlet. Recently, a novel approach based on cryotherapy was found to solve this problem, (Wang, Q.C. and J.P.T. Valkonen, 2008).

Indexing of Plants for SPFMV:

After heattherapy and meristem tip culture, the *in vitro* sixty two rooted plantlets two months old (fig. 2-C) subjected to RT-PCR analysis using specifically designed primers based on the conserved regions present in all viral strains obtained from the NCBI database. All detected samples were found to be SPFMV-free, a single band corresponding to the expected size of the amplified product of ~300 nucleotides. Figure (3-A) represents an example of the RT-PCR results obtained. Mein while, figure (3-B) showing an equal quality of RNA extracted from plants indicating that the absence of amplification in the treated samples is due to the absence of SPFMV and not to the quality of extracted RNA. Indexing method based on RT-PCR, although it is quite laborious and expensive (Fuglie, K.O. *et al*., 1999; Valverde, R.A., *et al*., 2007). However, it is sensitive.

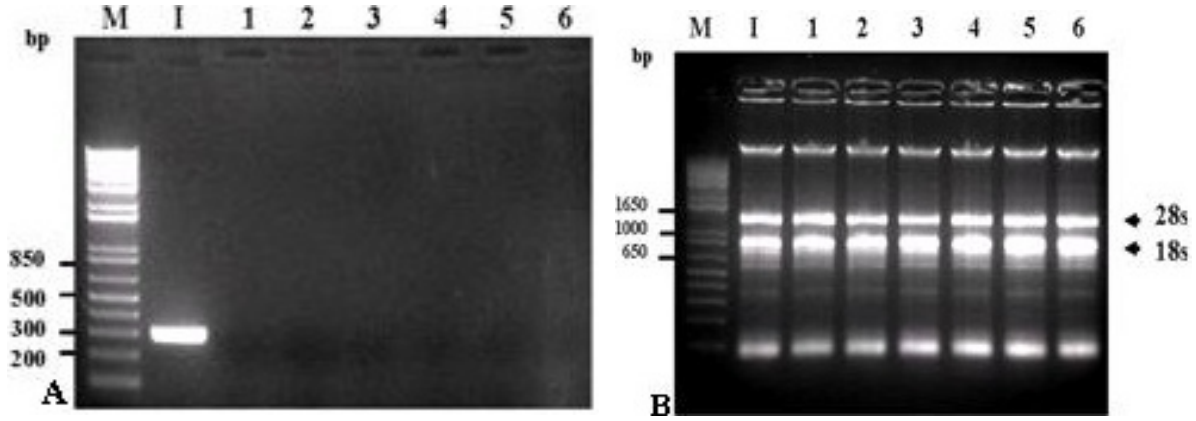

Fig. 3: **(A)** RT-PCR of sweetpotato samples after thermotherapy and meristem tip culture. Positive control (I), one kb plus molecular weight marker, invitrogen, USA (M) and reformed sweetpotato plants (1-6). **(B)** Aliquot of 2µg total RNA extracted from positive control and sweetpotato samples. Arrows indicate the 28s and 18s ribosomal RNA.

Dot-ELISA was routinely used to evaluate tissue culture materials over 2 years to confirm that the absence of SPFMV from sweetpotato plants is due to the freeing of plants form infection and not that the virus is under detection level.

Conclusion:

In the present study, we report the successful elimination of SPFMV from sweetpotato the Egyptian Abees cultivar by heattherapy and meristem tip culture of infected plants.

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