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Hot Water Treatment: An Efficient Method for Elimination of *Yam Mild Mosaic Virus* in *Dioscorea alata*

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Abstract

Greater yam (*Dioscorea alata*) is an important food crop in the Southern and North-Eastern states of India. Yams are prone to diseases. Among these, the *Yam mosaic virus* (YMV) and *Yam mild mosaic virus* (YMMV) belonging to the potyvirus group are widely prevalent. Hot water treatment is one of the methods of heat treatment in which the nodes are immersed in hot water for different time durations. With an aim to eliminate virus, hot water treatment of nodes has been tried to develop virus free planting materials through tissue culture. The vine cuttings (2-3 cm) of *Dioscorea alata* var. Sree Karthika with single node were treated with hot water at 32°C and 36°C for different time durations of 10, 20 and 30 minutes. The YMMV elimination was confirmed at regular intervals by RT-PCR with YMMV specific primers. Treatment of nodes at 36°C for 30 minutes was found to be the most efficient in virus elimination (90%), followed by 32°C for 30 minutes.

Key words: *Dioscorea alata*, hot water treatment, *yam mild mosaic virus*

Introduction

Yams (*Dioscorea* spp) are important tropical tuber crops that constitute staple food of millions of people in the tropics and subtropics. The most important cultivated edible yams are white Guinea yam (*Dioscorea rotundata* Poir.) and greater yam or water yam (*Dioscorea alata* L.). Greater yam has superior characteristics like high yield potential (especially under low to average soil fertility), ease of propagation, early vigour for weed suppression and shelf-life of tubers. In India, tubers of *D. alata* are consumed mostly in Southern and North-Eastern states. Their importance is greater than potato in some areas. However, yams are prone to infection by fungi, bacteria and viruses right from the seedling stage to harvesting and even later in storage. More than 15 viruses belonging to five different genera, namely, *Potyvirus*, *Macluravirus*, *Badnavirus*, *Cucumovirus* and *Potexvirus* are known to infect yams. However, *Yam mosaic virus* (YMV) and *Yam mild mosaic virus* (YMMV) belonging to potyvirus group are widely prevalent on yams in India.

The production of *in vitro* culture does not necessarily free plants from viruses. Heat therapy is one of the methods used for plant virus elimination, which includes shoot tip culture, chemotherapy, thermotherapy or various combinations of the above (Spiegel et al., 1993). Heat treatment is given through hot air or hot water, wherein hot water has proved better for dormant buds. Hot air treatment has generally given better elimination of viruses in actively growing shoots as well as better survival of the host. Hot water treatment (HWT) has been proposed by Caudwell (1966) to cure dormant

woody plant material from phytoplasmas. Subsequent work also showed the effectiveness of the treatment against phytoplasmas (Caudwell et al., 1990; Tassart-Subirats et al., 2003).

The present study aimed at the production of virus-free *D. alata* plants through *in vitro* propagation coupled with hot water therapy.

Materials and Methods

Explant selection

Dioscorea alata var. Sree Karthika (variety released from Centre Tuber Crops Research Institute (CTCRI) Thiruvananthapuram, India, plants grown in the shade house of CTCRI were used for the study. These plants which were produced from tubers of *in vitro* plants were found to be YMMV positive. The presence of YMMV in these plants was confirmed by RT-PCR.

Younger nodes (4-8 nodes from top) from the vines of *D. alata* plants were collected from the net house. These were washed thoroughly in running tap water for five minutes. The explants were then cut into small pieces with single nodes. As the initial step of surface sterilization, the explants were washed in 0.5% labolene (Fischer Scientific) for 10 minutes followed by washing in running tap water till all the traces of labolene was removed. Prior to the hot water treatment the explants were washed with distilled water. From these, a set of 30 nodes were wrapped in bags made of cheese cloth. Each set was taken for different temperature treatments for different time intervals. In this experiment, two different temperatures *viz.*, 32°C and 36°C were tried. The time duration for each temperature treatment was 10 minutes, 20 minutes and 30 minutes.

The nodes were divided into two sets. First set was taken for the treatment at 32°C and the second set at 36°C. These two sets were immersed in water bath (Julabo SW 21) set at 32°C and 36°C respectively. Of the 30 nodes, 10 nodes were taken out of the bags after 10 minutes from both treatments and were transferred to sterile beakers. Rest of the nodes inside the bags were again kept in water baths at respective temperatures and maintained for 10 more minutes. After 10 minutes, again 10 more nodes were taken from the bag. The remaining 10 nodes were again kept inside the water baths for another 10 minutes. Further sterilization was done inside the laminar air flow chamber. The nodes taken in separate sterile beakers were then sterilized using 0.08% $HgCl_2$ for two minutes followed by two sterile distilled water washes of one minute each. They were further sterilized with 70% alcohol for two minutes. Finally the explants were washed thoroughly with sterile distilled water three or four times for one minute duration for each wash and then transferred to liquid basal MS media supplemented with 0.2 mg l^{-1} Naphthalene Acetic Acid (NAA) and Benzyl Amino Purine (BAP) and activated charcoal (0.1%) in test tubes. After two weeks, the plants were transferred to solid MS media with the same combination. Total RNA from these plants were isolated after one month of growth and were indexed through RT-PCR.

Virus indexing

To test the presence of the virus in the *in vitro* grown plants, total RNA from the plants (leaves with small nodes) was isolated using Qiagen RNeasy Plant Mini kit. The isolated RNA was subjected to RT-PCR using Robust II RT-PCR kit (Finnzymes). The primers used were YV1-F, YV1-R and Oligo d(T). Twenty micro litres of the PCR reaction mix contained 2 μl of 10X buffer, 0.7 μl of $MgCl_2$, 0.5 μl of dNTPs, 0.07 μl of AMV Reverse Transcriptase, 0.5 μl of DNazyme, 0.25 μl of 40,000 U RNA Inhibitor, 9.98 μl of sterile distilled water and 3 μl of RNA. The cycling conditions were according to the conditions described by Mumford and Seal (1997); cDNA synthesis at 42°C for 30 minutes, initial denaturation at 94°C for 4 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. A final hold was maintained at 10°C. The PCR products were analysed on 1% agarose gel and the presence or absence of the virus was confirmed.

Multiplication of virus-free plants

The samples which were found negative for YMMV were further used for multiplication. The virus-free plants obtained were subcultured in solid MS media for one more month. After two subsequent subcultures, the *in vitro* plantlets were again virus indexed through RT-PCR as explained earlier.

Hardening

The confirmed virus-free plants were taken from the tubes without disturbing the roots, washed thoroughly in tap water and then treated with 0.1% bavistin for five minutes. The plants were again thoroughly washed in tap water to remove all traces of bavistin and then transferred to small plastic cups with holes and were covered with punched polythene bags and kept in shade house. After 10-15 days, the plants were transferred to bigger pots containing sand and soil in 1:1 ratio, with 0.25 kg of farmyard manure, followed by transfer onto the cement trough in the shade house.

Results

Explant selection

The explants treated at 32°C and 36°C for different time intervals were used for *in vitro* plantlet regeneration. Initially these explants grew well in liquid MS media supplemented with 0.2 mg l^{-1} NAA and BAP. After 2 weeks they were transferred to solid MS media with same composition. But, when they were transferred to solid media the survival rate of *in vitro* plantlets kept at 32°C was found to be less (~40%) when compared to those kept at 36°C (~70-80%).

Table 1. Percentage of virus-free *D. alata* var. Sree Karthika plants obtained after hot water treatment

Treatments	Virus-free plants (%) Time in minutes		
	10	20	30
Control	-	-	-
32°C	20	20	30
36°C	30	40	90

Virus indexing

The plants raised from the nodes treated at 32°C at different durations were found to be less effective in virus elimination when compared to those kept at 36°C (Table 1). The number of virus-free plants obtained after hot water treatment is given in Table 1. The plants raised from nodes treated at 36°C for 30 minutes produced the maximum number of virus-free plants (~90%) (Fig.1). Rest of the plants showed a ~260 bp fragment of partial CP of YMMV, which showed the presence of virus.

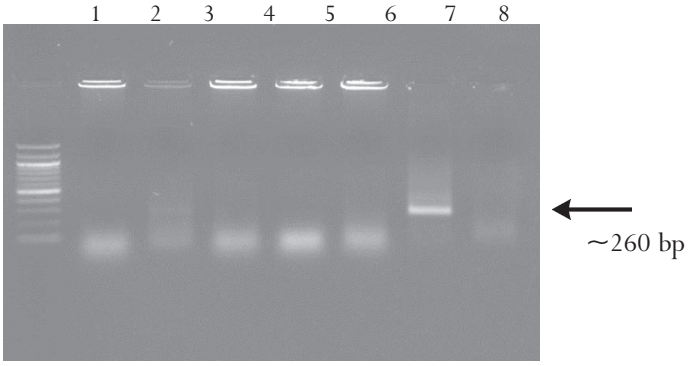

Fig. 1. Virus indexing of nodal culture of *D. alata* plants after hot water treatment through RT-PCR

Lane 1 : 100 bp ladder
Lane 2-6: Virus-free samples
Lane 7 : Positive control
Lane 8 : Negative control

Multiplication of virus-free plants

Fig. 2. Different stages of hardening
a) Virus-free plants hardened in small cups
b) The plants transferred to big pots
c) The plants which were finally transferred to cement trough in the shade house

The virus-negative plants were multiplied by sub culturing in the same media twice. These plants were again tested for the presence of the virus after one month of growth and were confirmed as virus-free plants through RT-PCR.

Hardening

The hardened virus-free plants were established successfully in small plastic cups as well as on transfer to sand-soil mixture in pots and were finally planted out in shade house (Fig. 2). The survival rate was about 80-90%. These plants were further used as the source material for large scale production of virus free yam plants.

Discussion

In general, when an infected plant is exposed to temperature between 30 to 40°C, the virus replication is inhibited and the young shoots continue to grow (Walkey, 1991) which do not necessarily show symptoms. The use of thermotherapy or hot water treatment (HWT) is recommended for the management of plant-parasitic nematodes and other pathogens for a range of planting material, especially vegetatively propagated crops including yams, *Dioscorea* spp. Over 100 years ago, Scots gardeners immersed bulbs in hot water before planting, thus being the first known users of heat for therapy of plants (Zandbergen, 1964). Mirla et al. (1986) also reported that sugarcane mosaic potyvirus was eliminated when treated with hot water for 4 days at 55, 56.5 and 57.5°C. In the United States, Quarantine Unit, Department of Agriculture also had used hot water treatment of sugarcane clones to eliminate pests and pathogens especially sugarcane mosaic caused by a potyvirus (Hurtt, 1996). Kunkel (1936) found that peach yellows was cured by either dry heat or hot water treatment and used both methods for curing 11 diseases of the yellow group, including aster yellows (Kunkel, 1945). Hot water was used earlier than hot air treatments and is used extensively against sugarcane diseases.

In the present study, it was found that through hot water treatment about 90% of virus-free plants could be made from virus positive plants. The most efficient temperature and duration for virus elimination was found to be 36°C for 30 minutes (~90%), which was followed by 32°C for 30 minutes (~30%). The survival rate of plants was also found to be more at 36°C. About 70-80% of the virus-free plants hardened in small cups could be successfully planted in cement trough in the shade house.

Thus the present study enables a simple and efficient method of virus eradication through hot water treatment of nodal segments. This will further help in large scale multiplication of healthy virus-free planting materials for distribution to the farmers and avoid the risk of introducing new viruses to new areas. This technique will be very much useful while exchanging germplasm to avoid introduction of other viruses.

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