Elimination of yam mosaic virus from yam using an optimized combination of meristem culture and thermotherapy

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Abstract

Vegetatively propagated plants like yam are prone to yield losses by viruses as infection tends to build up in successive cycles of propagation. This study aimed to eliminate yam mosaic virus (YMV) from yam using an optimized combination of thermotherapy and meristem culture. A protocol was optimized for shoot initiation, multiplication, and rooting from shoots of four yam varieties using Murashige and Skoog (MS) medium with growth regulators. A control at 26 ℃ and a thermo-treatment at 36, and 40 ℃ followed by meristem culture was used to eliminate YMV and verified using double antibody sandwich ELISA (DAS-ELISA) and reverse

transcription (RT)-PCR. Results showed that the highest explant initiation rate of 87.5% and 83.3% were obtained for variety Bulcha and Aw/Ar/005, respectively, on MS medium with 1.0 mg/L 6-benzylaminopurine (BAP) + 0.5 mg/L naphthaleneacetic acid (NAA). While the MS medium with BAP (0.5 mg/L) + NAA (0.25 mg/L) produced the highest initiation rate of 91.7% in variety Aw/Ar/001. The longest mean shoot length of 9.0±0.37 cm was recorded in Bulcha on MS medium with BAP (0.5 mg/L) + NAA (0.25 mg/L). The highest percentage of rooting was obtained on MS medium with 1.5 mg/L indole-3-butyric acid (IBA) + 0.5 mg/L NAA for variety Bulcha and variety Aw/Wo/011. The maximum YMV elimination (93.3–100.0%) was noted at 40 ˚C thermo-treatment for 20 days followed by meristem culture. No amplification of the 586 bp fragment of YMV was obtained by RT-PCR in all the plants that went through 40 ˚C thermotherapy. The results make significant contribution in improving yam production in many countries, particularly in Africa where YMV is a significant constraint.

Key words: *In vitro*, protocol optimization, screenhouse, virus-elimination, Yam, YMV

1. Introduction

The global human population is currently estimated at 8.0 billion and is projected to approach 10 billion by 2050 (UN 2022). In Sub-Saharan Africa, the current population is projected to double by 2050 (UN 2022) and triple by 2100 (Vollset et al. 2020). This will pose a significant challenge to food security, necessitating a substantial augmentation of existing infrastructure and food production to meet future demand (Giller 2020; Vollset et al. 2020). Consequently, the imperative arises for the development of highly productive varieties through conventional breeding and genetic engineering (Akhatar et al. 2022), as well as increasing the productivity of

existing varieties and implementing strategies for safeguarding against pathogens will become indispensable.

Yams (*Dioscorea* spp.) are monocotyledonous climbing crop species, classified in the family *Dioscoreaceae* and are represented by more than 800 species (Lebot 2009), of which ten species are used as edible crops (Mambole et al. 2014). Cultivated yam is ranked as the fourth most important root crop by production next to potato, sweet potato and cassava (Mignouna et al. 2005; Sukal et al. 2018). Ethiopia is the 5th major yam producer in the world (FAOSTAT 2017), and yam is cultivated for household food supply, income generation through marketing, and for its cultural and medicinal values (Bekele and Bekele 2020).

Like many other tuber crops, yams can be propagated through either seeds or tubers, which can lead to the accumulation of various pathogens, such as viruses, fungi, and bacteria (Sukal et al. 2018). Among these pathogens, viruses are particularly significant as they are recognized as one of the limiting factors for crop production (Eni et al. 2008; Kumar 2009; Abraham 2019). Yam mosaic virus (YMV) (genus *Potyvirus*, family *Potyviridae*) is among the most significant RNA viruses infecting yams globally (Luo et al. 2022). Potyviruses have single-stranded, positive-sense RNA with linear genomes (Gadhave et al. 2020). The monopartite genomes of the virions range in size from 9.4 to 11.0 kb and are flexuous, filamentous particles with length and width measurements of 680 to 900 nm and 11 to 20 nm, respectively (Yang et al. 2021; Pasin et al. 2022). YMV spreads naturally by vegetative reproduction of infected plant material, or through aphid vectors (Gadhave et al. 2020; Walker et al. 2021). It can also be mechanically transmitted through infected sap to other yam species, or indicator plants, like *Nicotiana benthamiana* (Ita et al. 2020; Monroy-Borrego and Steinmetz 2022).

In sub-Saharan Africa countries, like Ethiopia, despite the genetic diversity, and accessible cropland, the yam production has fallen from 200,000 annual productions in 1993 to 45,000 tons in 2020 (Adigoun-Akotegnon et al. 2020). YMV threatens food security and farmers' ability to generate sustainable income in the Southern Ethiopia. Recent studies showed that YMV is the prevalent (28.5%) viral pathogen widely infecting yam in the major growing areas of Southern Ethiopia (Gogile et al. manuscript accepted for publication by Heliyon).

Tissue culture is the most effective way to propagate plants, conserve genetic resources, eradicate viruses, and distribute plants internationally (Loyola-Vargas et al. 2018; Wang et al. 2018; Phillips and Garda 2019). Optimizing suitable protocols for *in vitro* multiplication and virus cleaning are believed to contribute substantially to improving the availability of healthy planting materials in adequate quantities for resource-poor farmers. This will also facilitate the efficient exchange of healthy germplasms and preventing the spread of viruses into new geographic areas. In this study, it is presented the optimization of an effective multiplication protocol for inducing shoots and roots from *in vivo* apical yam buds and eliminate YMV using thermotherapy followed by meristem culture to produce YMV-free yam seedlings in Ethiopia.

2. Materials and methods

2.1 Sample collection and mother plants establishment

Yam fields located along main and rural roads in southern Ethiopia were randomly surveyed at intervals of approximately 4-8 km between October and November during the cropping seasons of 2021 and 2022. The major yam cultivating areas that cumulatively share over 70% were identified (Gogile et al., unpublished data). Yam tubers were collected from four randomly selected yam-cultivating locations, namely, Wolaita (Gununo), Goffa (Kucha), Hawassa Agricultural Research Center (HARC), and Areka Agricultural Research Center (AARC)

(Supplementary Table 1). These tubers were maintained in both field and screenhouse conditions at AARC. The yam tubers collected for the experiment were from farmers' and researchers' preferred species of Dioscorea rotundata (varieties Bulcha, n = 15), Aw/Ar/001 (n = 50), and Aw/Ar/005 (n = 35), and Dioscorea alata (variety Aw/Wo/011, n = 40) plants. To eliminate any potential pests such as mealybugs, mites, and aphids, the tubers were disinfected with 10% sodium hypochlorite (NaClO; 4% active chlorine) for 30 minutes before planting them in a mixture of soil and organic compost at a 3:1 ratio, respectively, in plastic pots (6.5 inches in width x 6.75 inches in height). Each plant was assigned a unique identification number and grown in an insect-free quarantine screenhouse at a temperature of 25 ± 2°C, relative humidity of 50% - 60%, and a 12-hour light-dark cycle for three months. Management practices such as irrigation, harvesting weeds, and fertilizer additions were done, and any virus-like disease symptom development was inspected.

2.2 Mother plants screening for the presence of YMV

Yam plants were tested for the presence of YMV using double antibody sandwich (DAS)-ELISA (Clark and Adams 1977) with virus specific antibodies and alkaline-phosphatase conjugates obtained from Leibniz Institute DSMZ Germany Collection of Microorganisms and cell cultures (GmbH) (DSMZ, RT-1142) following the manufacturer's instructions with minor modifications, in which 150 µL potyvirus-specific monoclonal antibody and rabbit anti-mouse conjugate were used instead of 200 µL. YMV-free yam samples from previous study by Gogile et al. (manuscript accepted for publication by Heliyon), were used as negative control to ensure the success of the assay. After following all the steps in manufacturers, the microplate wells were visually observed for yellow colour development for the presence of virus and no colour change (white colour) for the absence. The optical density values were measured with a

Spectrophotometer (DR-200BS Microplate reader (Diatek)) at an absorbance of 405 nm to quantify the ELISA results. A sample was taken as positive when the value of absorbance at 405 nm was greater than twice the mean absorbance reading of the negative control as described by Silva et al. (2015; 2019). For further screening, the total RNA was extracted from fresh leaves using the RNeasy® Mini Kit (cat. Nos. 74104 and 74106, QIAGEN) following the manufacturer's instructions. The RNA integrity and quantity were checked by electrophoresis using a 1% agarose gel and NanoDrop 2000 spectrophotometer. The total volume of the RT-PCR amplification was 25 µL consisting of 2 µL of extracted RNA, 2 µL of dNTPs mixes (10 mM), 5 µL of 5 x M.MLV RT buffer, 0.5 µL of each primer, 0.5 µL of M-MLV Reverse Transcriptase (RT) (Promega, USA), 1 µL of Dream Tag DNA polymerase, and 13.5 µL of nuclease-free water. The one-step RT-PCR was performed using YMV-specific primers (YMV-FP 5'-ATCCGGGATGTGGACAATGA -3', and YMV-RP 5'- TGGTCCTCCGCCACATCAAA -3') targeting the coat protein (CP) and 3' untranslated region (3'UTR) -coding regions as described by Silva et al. (2015). YMV-free yam sample from the previous study by Gogile et al. (manuscript accepted for publication by Heliyon), was used as a negative control. The amplified products (10 µL) were analyzed by agarose gel electrophoresis using 1% (w/v) agarose and GeneRulerTM 100 bp DNA ladder. Virus-free plants were kept in the screenhouse for comparative testing and *in vitro* protocol optimization, whereas, the YMV-positive plants were employed as explant sources for the virus-cleaning procedure through thermotherapy and meristem culture.

2.3 *In vitro* shoot initiation and proliferation

Apical or axillary buds from three *D. rotundata* varieties, namely Bulcha, Aw/Ar/001, and Aw/Ar/005, and one *D. alata* variety, Aw/Wo/011, were used for the *in vitro* propagation

protocol optimization. Shoots with approximately 1.0 to 2.5 cm in length containing one bud were harvested from screenhouse-grown plants after three months of vegetative growth. Thirty-six (36) cuttings per variety (either apical or axillary buds) were cut with scissors and kept in a dry clean container and variety names were recorded. Shoots were washed with soap water, rinsed, and treated with 75% ethanol for 5 min, followed by three times with autoclaved double distilled water (ddH2O) and treatment with 10% sodium hypochlorite (NaClO) solution with two drops of a Tween-20 for 15 min. Finally, the shoots rinsed three times with sterile ddH2O. Shoots were carefully trimmed at their ends and inoculated in the initiation medium (Mignouna et al. 2009). The yam basic medium (YBM) was based on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), containing 30 g/L sucrose, 6-benzylaminopurine (BAP) at concentrations of 0.0, 0.5, 1.0, 2.0 and 3.0 mg/L and naphthalene acetic acid (NAA) at concentrations of 0.0, 0.25 and 0.5 mg/L, 8 g/L agar at pH 5.8 (Supplementary Table 2). YBM medium was used for both initiation and shoot multiplication as described by Amiri and Mohammadi (2019). A single surface sterilized shoot was cultured per culture jar. For each yam variety, twelve shoots were micropropagated for each medium treatment and the experiment was triplicated. Cultures were grown at 25±1 °C under a photoperiod of 16/8 h (light/dark) as described by Mignouna et al. (2009) and Wang et al. (2018). Shoots were considered regenerated when fresh leaves appeared and continued to grow into a plant. Cultures were placed in a completely randomized design (CRD) and the percentage of shoot initiation was recorded after four weeks. The percentage of shoot initiation was calculated as the total number of initiated explants over the total number of explants inoculated × 100% (Abide et al. 2022). Days to shoot initiation was observed for 20 successive days and recorded. After six weeks on initiation media, the regenerated shoots were transferred onto shoot proliferation MS medium supplemented with

BAP (0.0, 0.5, 1.0, 2.0, and 3.0 mg/L) and NAA (0.0, 0.25, 0.5 mg/L) concentrations and combinations (Supplementary Table 2) and kept in the growth chamber at 25±1 °C under a photoperiod of 16/8 h (light/dark). Shoots were sub-cultured to fresh medium every three weeks for three times to produce multiple clones (Ita et al. 2020; Abide et al. 2022). Then, shoot proliferation (shoots produced per explants inoculated) and shoot length (cm) was recorded after a month from the last sub-culturing as described in Phillips and Garda (2019).

2.4 *In vitro* rooting

Plantlets obtained from shoot proliferation media were transferred to a hormone free media for two weeks for rejuvenation and avoiding the effect of multiplication hormones on rooting. Well-developed shoots with 4-5 cm shoot length were transferred onto rooting medium (RM) of MS containing 30 g/L sucrose supplemented with IBA at 0.0, 0.5, 1.5, and 2 mg/L and NAA at 0.0, 0.5, and 1 mg/L (Supplementary Table 2), and 25 mg/L ascorbic acid and solidified with 8.0 g/L agar at pH 5.8. The plantlets were cultured under the same conditions as the stock cultures for a month. Then rooting (the total number of rooted shoots over the total number of shoots transferred to RM × 100%) and root lengths (cm) were recorded as described in Amiri and Mohammadi (2019) and Abide et al. (2022).

2.4 YMV elimination using thermotherapy and shoot apex culture

Apical or axillary buds (1.0 to 2.5 cm in length) comprising a bud were excised from five-week-old screenhouse plants of YMV-infected varieties. The excised buds were then cultured on MS medium with BAP and NAA hormones specific to each variety, which were optimized earlier in section 2.3. Plants were established on their respective media for a month before heat treatment. Two different temperatures: (a) 36 ˚C, and (b) 40 ˚C were used for the heat treatment. A 26 ˚C temperature was used as a control. The 36 ˚C and 40 ˚C temperatures were achieved by gradually

increasing the temperature with 2 ˚C per day, from 26 ˚C, and kept in a respective thermotherapy chamber for 20 days (Mignouna et al. 2009). A total of 24–30 plants from each variety (eight to ten plants per each temperature treatment) were used. Following the thermotherapy treatments, all the plants were kept at a constant temperature of 25±1 °C for a month. Under laminar flow, shoots were transferred into a sterile container using sterilized forceps and rinsed three times with sterile ddH2O. A single shoot was placed under a dissecting microscope (the microscope was sprayed and cleaned with 70% alcohol before use) and leaf primordia was cut using a sterile scalpel. The meristematic dome (0.1 - 0.2 cm) was excised and cultured on MS medium with BAP and NAA hormones specific to each variety, which were optimized earlier in section 2.3 for three months (Mignouna et al. 2009; Agbidinoukoun et al. 2013). The experiment was triplicated and the *in vitro* plantletes were evaluated for shoot regeneration and survival rate. Furthermore, matured and young leaf parts were evaluated for the elimination of YMV using DAS-ELISA and RT-PCR as described earlier in section 2.2. Then the shoots were sub-cultured every three weeks during 45 days. Well-developed shoots with 4-5 cm shoot length were transferred onto rooting media supplemented with variety-specific IBA and NAA concentrations and combinations optimized earlier in section 2.4. Plants were grown in the rooting media for a month under the same conditions as the stock cultures. The growth responses of the plantlets including the percentage of rooting, shoot length (cm), root length (cm), the number of leaves per explants, and the number of roots per explant were recorded.

2.5 Yam plants establishment and screening for the elimination of YMV

The well developed plantlets (shoot length ≥5 cm) were removed from the rooting medium and after washing thoroughly in running tap water eight to ten rooted plantlets per variety were transferred to cross-sectionally cut plastic water bottles (2L), filled with autoclaved red soil, sand,

and manure mix at 2:1:1 ratio, respectively, and grown further in an insect-proof screenhouse under a controlled temperature of 25±1 ºC and a photoperiod of 12 h (light/dark). The plastic bottles were partially covered to maintain higher humidity and gradually opened once the plantlets had adapted to the environment (Fig 2, K-L). After a month, the plantlets were transferred onto a 30 cm pots filled with autoclaved red soil, sand, and manure as described above, and the development of symptoms was periodically observed. The experiments were triplicated and eight to ten samples per treatment were used. The mean plant height (cm) and mean number of leaves in the screenhouse were recorded. The acclimatized plants were screened for the elimination of YMV using DAS-ELISA and the result was confirmed by RT-PCR after four months of acclimatization. The procedures followed are presented in the schematic diagram in Supplementary Fig. 1. Quantitative data were subjected to SPSS software (version 16) and the results of the replicates were represented as mean and standard error (SE).

3. Result

3.1 Screening for YMV in mother plant using DAS-ELISA and RT-PCR

The yam varieties Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011 established in the screenhouse showed typical viral symptoms, such as mosaics through visual inspection. However, Bulcha variety appeared healthy and did not show any symptom. On screening mother plants for YMV using DAS-ELISA and RT-PCR samples from Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011 reacted positively on DAS-ELISA, whereas samples from Bulcha were negative. Similarly, samples from Bulcha tested negative on RT-PCR, while all the samples from Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011 gave a positive result.

3.2 *In vitro* protocol optimization for shoot initiation and proliferation

The results from *in vitro* shoot initiation showed variations among the treatments and the varieties (Table 1; Fig. 1 a, b). Explants from Bulcha started to initiate shoot within 6 – 15 days of post culturing, while variety Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011 resulted in shoot initiation in 8 – 16, 8 – 20, and 6 – 17 days of post culturing, respectively (Table 1). The highest mean explant regeneration of 87.50% and 62.50% were obtained for variety Bulcha on MS medium supplemented with BAP (1.0 mg/L) + NAA (0.5 mg/L) and BAP (1.0 mg/L) + NAA (0.25 mg/L), respectively. In variety Aw/Ar/001, the highest explant initiation rate of 91.67% and 66.67% were recorded on MS medium supplemented with BAP (0.5 mg/L) + NAA (0.25 mg/L) and BAP (0.5 mg/L) + NAA (0.5 mg/L), respectively. Whereas, in variety Aw/Ar/005, the highest mean explant initiation rate of 83.33% and 62.50% were obtained on MS medium supplemented with BAP (1.0 mg/L) + NAA (0.5 mg/L), and BAP (2.0 mg/L) + NAA (0.25 mg/L), respectively. On the other hand, in variety Aw/Wo/011, the highest mean explant initiation rate of 79.17% and 66.67% were obtained on MS medium supplemented with BAP (2.0 mg/L) + NAA (0.5 mg/L), and BAP (2.0 mg/L) + NAA (0.25 mg/L), respectively (Table 1).

The result also showed that the medium concentration of BAP (0.5 mg/L) and NAA (0.25 mg/L) produced the highest number of shoot proliferation (4.17±0.35) and the longest shoot length (8.97±0.37 cm) in Bulcha. The other combinations of BAP (1.0 mg/L) + NAA (0.5 mg/L) also generated higher number of shoot proliferation (4.13±0.32) and longer shoot length (5.33±0.43 cm) in Bulcha. In variety Aw/Ar/001, the highest mean number of shoot proliferation (5.83±0.03) was recorded in BAP (0.5 mg/L) + NAA (0.5 mg/L) while, the longest shoot length (4.33±0.58 cm) was recorded on MS medium supplemented with BAP (0.5 mg/L) + NAA (0.25 mg/L). Whereas, in variety Aw/Ar/005, the highest number of shoot proliferation (4.57±0.09)

and the longest shoot length (6.33±0.67 cm) was recorded on MS medium supplemented with BAP (0.5 mg/L) + NAA (0.5 mg/L) and BAP (1.0 mg/L) + NAA (0.5 mg/L), respectively. The MS medium supplemented with BAP (1.0 mg/L) + NAA (0.5 mg/L) promoted higher number of shoot proliferation (4.33±0.45), while BAP (2.0 mg/L) + NAA (0.5 mg/L) enhanced the longest shoot length (5.00±0.58 cm) in Aw/Wo/011. Overall, the lowest mean shoot regeneration, shoot proliferation and shoot length was recorded on MS medium without plant growth regulators (PGRs) in all the varieties (Table 1; Fig. 1 c, d).

Table 1. The effect of different concentrations and combinations of plant growth hormones on explant regeneration, shoot proliferation, and shoot length

Varieties (N)	PGRs (mg/L)		shoot regeneration	shoot initiation time (number of days)	Shoot proliferation	Shoot length
	BAP	NAA	rate (%)		(Shoots/explant) (mean±SE)	(cm) (mean±SE)
Bulcha (12)	0.0	0.0	29.17^{e}	15	1.90±0.62^{d}	2.27±0.07^{e}
		0.25	33.33^{e}	14	1.67±0.67^{d}	2.40±0.10^{e}
		0.5	33.33^{e}	14	2.40±0.21^{c}	4.17±0.28^{c}
	0.5	0.0	37.50^{e}	15	3.57±0.09^{b}	3.17±0.03^{d}
		0.25	50.00^{c}	13	**4.17±0.35^{a}**	**8.97±0.37^{a}**
		0.5	54.17^{c}	13	3.40±1.25^{b}	5.27±0.34^{b}
	1.0	0.0	41.67^{d}	14	3.67±0.35^{b}	4.23±0.32^{c}
		0.25	62.50^{b}	10	3.43±0.54^{b}	4.17±0.30^{c}
		0.5	**87.50^{a}**	10	4.13±0.32^{a}	5.33±0.43^{b}
	2.0	0.0	58.33^{b}	13	3.43±0.12^{b}	4.77±0.12^{b}
		0.25	50.00^{c}	8	2.50±0.15^{c}	3.00±0.00^{d}
		0.5	54.17^{c}	8	3.40±0.64^{b}	5.00±0.58^{b}
	3.0	0.0	41.67^{d}	10	2.10±0.58^{c}	4.67±0.88^{b}
		0.25	50.00^{c}	6	1.73±0.30^{d}	2.67±0.67^{e}
		0.5	45.83^{d}	6	1. 70±0.06^{d}	2.33±0.67^{e}
Aw/Ar/001 (12)	0.0	0.0	29.17^{f}	16	1.50±0.29^{e}	1.20±0.06^{d}
		0.25	33.33^{e}	16	1.37±0.20^{e}	2.67±0.33^{c}
		0.5	33.33^{e}	13	1.87±0.15^{d}	3.00±0.00^{c}
	0.5	0.0	37.50^{e}	15	1.73±0.28^{d}	3.33±0.33^{c}
		0.25	**91.67^{a}**	12	4.30±0.59^{b}	4.33±0.58^{b}
		0.5	66.67^{b}	12	**5.83±0.03^{a}**	4.00±0.67^{b}
	1.0	0.0	41.67^{d}	15	2.30±0.90^{d}	3.67±0.67^{b}
		0.25	50.00^{c}	12	2.00±0.58^{d}	3.67±0.88^{b}
		0.5	50.00^{c}	12	2.80±0.35^{c}	4.67±0.33^{a}
	2.0	0.0	41.67^{d}	11	2.87±0.23^{c}	4.00±0.00^{b}
		0.25	50.00^{c}	10	1.63±0.15^{e}	3.00±0.00^{c}
		0.5	37.50^{e}	10	3.10±0.40^{c}	**5.00±0.58^{a}**
	3.0	0.0	45.83^{d}	11	3.10±0.35^{c}	2.67±0.33^{c}
		0.25	50.00^{c}	8	2.70±0.35^{c}	3.67±0.33^{b}
		0.5	45.83^{d}	8	0.80±0.40^{f}	2.33±0.67^{c}
Aw/Ar/005 (12)	0.0	0.0	17.50^{f}	20	1.27±0.18^{e}	2.00±0.00^{e}
		0.25	33.33^{e}	18	1.30±0.17^{e}	2.33±0.33^{e}
		0.5	33.33^{e}	18	1.87±0.23^{d}	3.00±0.00^{d}
	0.5	0.0	37.50^{e}	16	3.10±0.47^{c}	3.67±0.33^{c}
		0.25	58.33^{b}	15	3.03±0.22^{c}	4.67±0.67^{b}
		0.5	58.33^{b}	14	**4.57±0.09^{a}**	4.33±0.33^{c}
	1.0	0.0	41.67^{d}	15	3.00±0.67^{c}	4.00±0.58^{c}
		0.25	50.00^{c}	13	2.57±0.29^{d}	3.67±0.33^{c}
		0.5	**83.33^{a}**	13	3.33±0.33^{c}	**6.33±0.67^{a}**
	2.0	0.0	50.00^{c}	12	3.03±0.34^{c}	4.00±0.00^{c}
		0.25	62.50^{b}	8	2.00±0.00^{d}	3.00±0.00^{d}
		0.5	50.00^{c}	8	3.33±0.33^{c}	5.00±0.58^{b}
	3.0	0.0	45.83^{c}	10	3.87±0.19^{b}	3.67±0.33^{c}
		0.25	50.00^{c}	6	3.40±0.20^{c}	4.67±0.33^{b}
		0.5	45.83^{c}	8	1.00±0.58^{e}	2.33±0.67^{e}
Aw/Wo/011(12)	0.0	0.0	29.17^{f}	17	1.33±0.33^{d}	1.07±0.03^{d}
		0.25	33.33^{e}	15	1.43±0.30^{d}	1.13±0.03^{d}
		0.5	33.33^{e}	14	1.93±0.09^{c}	1.67±0.23^{d}
	0.5	0.0	37.50^{e}	15	2.53±0.03^{c}	3.00±0.00^{c}
		0.25	50.00^{c}	12	3.00±0.00^{b}	4.33±0.33^{b}
		0.5	54.17^{c}	12	1.97±0.24^{c}	4.00±0.00^{b}
	1.0	0.0	41.67^{d}	11	2.07±0.30^{c}	3.67±0.33^{b}
		0.25	50.00^{c}	10	2.33±1.20^{c}	3.67±1.45^{b}
		0.5	54.17^{c}	10	**4.33±0.45^{a}**	4.67±0.33^{a}
	2.0	0.0	58.33^{c}	11	2.77±0.39^{c}	3.00±0.00^{c}
		0.25	66.67^{b}	9	2.57±0.28^{c}	3.60±0.40^{b}
		0.5	**79.17^{a}**	8	4.27±0.49^{b}	**5.00±0.58^{a}**
	3.0	0.0	54.17^{c}	10	3.33±0.88^{b}	3.33±0.33^{c}
		0.25	45.83^{d}	7	2.60±0.55^{c}	3.67±0.67^{b}
		0.5	41.67^{d}	6	2.53±0.60^{c}	4.00±0.58^{b}

N: no of samples tested for each variety, PGR: plant growth regulators, BAP: 6-benzylaminopurine, NAA: naphthalene acetic acid, SE:standard error. The best treatment for each variety is indicated by a bold values.

3.3 *In vitro* root development for yam varieties

Rooting occurred two to three weeks after culturing on RM and showed significant variation among the different varieties and treatments applied (Table 1; Fig. 1 e, f). Among the various treatments, the MS medium supplemented with IBA (1.5 mg/L) + NAA (0.5 mg/L), and IBA (1.5 mg/L) + NAA (0.25 mg/L) resulted in the maximum mean root formation of 88.89% and 62.50%, respectively, for the Bulcha variety. In addition, the Bulcha variety exhibited the longest root length of 5.87±0.22 and 6.33±0.43 cm on the same media combinations, respectively. While variety Aw/Ar/001 produced the highest percentage of root (85.71%) and the longest mean root length (5.00±0.00 cm) on MS medium supplemented with IBA (1.5 mg/L) + NAA (0.25 mg/L) and IBA (1.5 mg/L) + NAA (0.5 mg/L), respectively. Similarly, for variety Aw/Ar/005, the highest percentage of root (87.50%) and the longest mean root length (4.00±0.00 cm) were recorded on MS medium supplemented with IBA (1.5 mg/L) + NAA (0.25 mg/L). In variety Aw/Wo/011, IBA (1.5 mg/L) + NAA (0.5 mg/L) resulted in the highest percentage of rooting (79.17%), while the same concentration of IBA (1.5 mg/L) with halved NAA (0.25 mg/L) generated the longest root of 4.33±0.33 cm.

Fig. 1. *In vitro* shoot regeneration, proliferation, and rooting from apical/axillary buds of yam varieties in MS media supplemented with PGRs. (a, b) shoot initiation after four weeks; (c, d) shoot proliferation after two months ; (e, f) rooting after a month

Table 2. *In vitro* rooting percent and root length for different yam varieties

Varieties (N)	PGRs (mg/L)		Rooting (%)	Root length (cm) Mean±SE
	IBA	NAA		
Bulcha (12)	0.0	0.0	16.67^{f}	1.97±0.30^{e}
		0.5	25.00^{e}	2.10±0.06^{e}
		1.0	41.67^{d}	2.37±0.12^{d}
	0.5	0.0	45.83^{d}	2.45±0.20^{d}
		0.25	54.17^{c}	4.73±0.09^{b}
		0.5	50.00^{c}	4.23±0.32^{c}
	1.5	0.0	50.00^{c}	2.77±0.03^{d}
		0.25	62.50^{b}	**6.33±0.43^{a}**
		0.5	**88.89^{a}**	5.87±0.22^{a}
	2.0	0.0	45.83^{d}	3.57±0.78^{c}
		0.25	58.33^{c}	4.00±0.58^{c}
		0.5	45.83^{d}	4.20±0.20^{c}
Aw/Ar/001 (12)	0.0	0.0	15.38^{f}	0.67±0.33^{e}
		0.25	26.67^{e}	1.33±0.33^{d}
		0.5	33.33^{d}	1.70±0.15^{d}
	0.5	0.0	36.67^{d}	3.00±0.00^{c}
		0.25	46.67^{c}	3.33±0.33^{c}
		0.5	56.67^{b}	3.67±0.67^{b}
	1.5	0.0	40.00^{c}	3.67±0.88^{b}
		0.25	**85.71^{a}**	4.67±0.33^{a}
		0.5	60.00^{b}	**5.00±0.00^{a}**
	2.0	0.0	47.33^{c}	3.67±0.33^{b}
		0.25	46.67^{c}	4.00±0.58^{b}
		0.5	43.33^{c}	3.67±0.33^{b}
Aw/Ar/005 (12)	0.0	0.0	12.50^{f}	1.33±0.88^{d}
		0.25	29.17e	1.67±0.33^{d}
		0.5	37.50^{e}	1.67±0.33^{d}
	0.5	0.0	33.33^{e}	2.33±0.33^{c}
		0.25	45.83^{d}	3.67±0.33^{a}
		0.5	54.17^{c}	3.73±0.37^{a}
	1.5	0.0	37.50^{e}	3.00±0.00^{b}
		0.25	**87.50^{a}**	**4.00±0.00^{a}**
		0.5	66.67^{b}	**4.00±0.00^{a}**
	2.0	0.0	33.33^{e}	3.00±0.00^{b}
		0.25	50.00^{c}	3.67±0.33^{a}
		0.5	54.17^{c}	3.80±0.42^{a}
Aw/Wo/011 (12)	0.0	0.0	20.83^{f}	1.00±0.00^{e}
		0.25	29.17^{e}	1.57±0.32^{d}
		0.5	29.17^{e}	1.77±0.39^{d}
	0.5	0.0	37.50^{d}	2.27±0.34^{c}
		0.25	54.17^{b}	3.67±0.33^{a}
		0.5	45.83^{c}	3.33±0.33^{b}
	1.5	0.0	50.00^{b}	2.70±0.30^{c}
		0.25	54.17^{b}	**4.33±0.33^{a}**
		0.5	**79.17^{a}**	3.67±0.33^{a}
	2.0	0.0	41.67^{c}	2.67±0.33^{c}
		0.25	45.83^{c}	4.17±0.17^{a}
		0.5	50.00^{b}	3.97±0.58^{a}

N: no of samples tested for each variety, PGR: plant growth regulators, IBA: indol butric acid, NAA: naphthalene acetic acid, SE:standard error. The bold values indicates the best treatment for each variety .

3.4 Shoot regeneration, survival rate and YMV elimination after thermo-treatment under *in vitro* and screenhouse conditions

The YMV elimination experiment was carried out on Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011. The variety Bulcha was included for the thermotherapy experiment as a control. The *in vitro* shoot initiation frequency decreased from 100% to 25% for Aw/Ar/001, 93% to 20% for Aw/Ar/005, and 80% to 17% for Aw/Wo/011 as the thermo-treatment temperature increased from 26 ˚C (control) to 40 ˚C, respectively (Table 3). The YMV elimination efficiency increased from 21% to 100% in Aw/Ar/001, 30% to 100% in Aw/Ar/005, and 17% to 97% in Aw/Aw/011 as the *in vitro* thermo-treatment temperature increased from 26 to 40 ˚C. The maximum YMV elimination (100.0%) was noted in Aw/Ar/001, and Aw/Ar/005 from *D. rotundata* species, while 96.67% and 93.33% were obtained in Aw/Wo/011 from *D. alata* species at 40 ˚C thermo-treatment from *in vitro* and screenhouse, respectively. On the other hand, 36 ˚C thermo-treatments eliminated half or more than half percentage (50.00, 66.67, and 75.00) of YMV in Aw/Wo/011, Aw/Ar/001, and Aw/Ar/005, respectively. The lowest YMV elimination (16.67%, 25.00%, and 10.00%) was recorded at 26 ˚C in Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011, respectively.Variety Bulcha as a negative control, did not produce significant values in all the parameters considered (Table 3).

Table 3. The effect of an optimized thermo-treatment and meristem culture on YMV elimination and survival rate in tested yam varieties under *in vitro* and screenhouse conditions

Varieties	Temperature (˚C) variation	No of samples treated	Three-month post thermo-treated (*in vitro*)		4-months of acclimatization (Screenhouse)	
			Shoot initiation (%)	Virus elimination efficiency (%)	Plantlets survival rate (%)	Virus elimination efficiency (%)
Aw/Ar/001	26	8	100.00[a]	20.83[c]	83.33[a]	16.67[c]
	36	8	41.67[b]	70.83[b]	61.11[b]	66.67[b]
	40	8	25.00[c]	100.00[a]	50.00[c]	100.00[a]
Aw/Ar/005	26	8	93.33[a]	30.00[c]	87.50[a]	25.00[c]
	36	8	50.00[b]	80.00[b]	66.67[b]	75.00[b]
	40	8	20.00[c]	100.0[a]	58.33[c]	100.00[a]
Aw/Wo/011	26	10	80.00[a]	16.67[c]	76.67[a]	10.00[c]
	36	10	40.00[b]	50.00[b]	56.67[b]	50.00[b]
	40	10	16.67[c]	96.67[a]	46.67[c]	93.33[a]
Bulcha*	26	10	93.33[a]	100.00[a]	80.00[a]	100.00[a]
	36	10	90.00[a]	100.00[a]	80.00[a]	100.00[a]
	40	10	90.00[a]	100.00[a]	76.67[a]	100.00[a]

Means with significant differences ($P \leq 0.05$) are represented by different letters for each variety. (*) virus-negative control

3.5 Evaluation of regenerated plants *in vitro* and in the screenhouse

Performance evaluation of thermo-treated yam varieties *in vitro* and screenhouse conditions were summarized in Fig. 2, A–H and I–P, respectively. The *in vitro* analysis indicated significantly higher mean shoot length (cm) (16.76 ±0.33), mean root length (cm) (14.67±0.33), and mean number of roots (16.00±0.58) in thermo-treated YMV-free Aw/Ar/005 as compared to non-thermotreated YMV positive counterparts (Table 4). On the other hand, the screenhouse results showed significantly higher (P<0.05) mean plant height (32.08±1.3, 48.37±1.84, and 23.82±1.51 cm) in Aw/Ar/001, Aw/Ar/005, and Aw/Wo/011, respectively), against the non-treated (28.88±0.88, 39.19±1.81 and 15.60±0.24 cm). Moreover, the thermo-treated variety Aw/Ar/005

and Aw/Wo/011 produced significantly more number of leaves (35.33±0.33 and 15.00±0.58) as compared to the non-treated (32.33±0.33 and 11.67±0.33) in the screenhouse (Table 4).

Verification testing of the acclimatized plants by DAS-ELISA followed by RT-PCR showed that there was no amplification of the 586-base pair (bp) segment of the YMV-CP and 3' UTR coding region in any of the samples produced by 40 ˚C thermo treated (Supplementary Fig. 2, E–J), indicating that the samples are YMV-free. On the other hand, except one - to - two samples from a 26 ˚C control meristem culture (Supplementary Fig. 2, A–D) and almost half of the samples treated with 36 ˚C heat, produced positive results on the DAS-ELISA and RT-PCR testing (data not shown).

Table 4 Performance of thermo-treated (YMV-free) and non-treated (YMV-infected) yam varieties under *in vitro* and screenhouse conditions. Mean±SE represents the value of three replicates.

Varieties	Type of plants	Plant No	*In vitro* (after one month of treatment)					Screenhouse (after 4-months of acclimatization)	
			Shoot length (cm) Mean±SE	No of leaves Mean±SE	Rooting (%)	Root length (cm) Mean±SE	No of roots Mean±SE	Plant height (cm) Mean±SE	No of leaves Mean±SE
Aw/Ar/001	YMV-free	8	9.67±0.33^{a}	12.00 ±0.58^{a}	95.83 ^{a}	12.33 ±0.57^{a}	7.00 ±0.58^{a}	34.08±1.3^{a}	18.00±0.00^{a}
	YMV-infected	8	9.63 ±0.36^{a}	11.33 ±0.33^{a}	91.67 ^{a}	10.97 ±0.90^{a}	6.33 ±0.33^{a}	28.88±0.88^{b}	17.33±0.33^{a}
Aw/Ar/005	YMV-free	8	16.76±0.33^{a}	10.00 ±0.58^{a}	83.33 ^{a}	14.67±0.33^{a}	16.00±0.58^{a}	48.37±1.84^{a}	35.33±0.33^{a}
	YMV-infected	8	12.41 ±0.48^{b}	9.67 ±0.33^{a}	83.33 ^{a}	10.66 ±0.43^{b}	12.33 ±0.33^{b}	39.19±1.81^{b}	30.33±0.33^{b}
Aw/Wo/011	YMV-free	8	9.48 ±0.45^{a}	7.00 ±0.00^{a}	87.50 ^{a}	12.82 ±0.16^{a}	5.67 ±0.33^{a}	23.82±1.51^{a}	15.00±0.58^{a}
	YMV-infected	8	8.48 ±0.56^{a}	6.00 ±0.58^{a}	83.33 ^{a}	10.10 ±0.11^{a}	4.67 ±0.33^{a}	15.60±0.24^{b}	11.67±0.33^{b}
Bulcha*	YMV-free	12	10.77±0.37	11.34±0.13	90.00	12.13±0.03	14.00±0.76	35.81±1.5	32.25±0.33

SE:standard error. Means with significant difference($P \leq 0.05$) are represented by different letters for each variety. (*) virus free control


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Fig. 2. Performance evaluation of thermo-treated yam varieties *in vitro* and screenhouse conditions. (A–C) and (E–G): shoot elongation and shoot multiplication of thermo-treated Aw/Ar/001, Aw/Ar/005, and Aw/Aw/011, respectively; (D–H): non-thermo-treated (control); (I–J): *In vitro* rooted shoots; (K–L): rooted plantlets acclimatization in plastic bottles for gradual adaptation; (M–O): the thermo-treated plants adapted in the screenhouse after 4 months, and (P) non-thermo-treated (control).

Discussion

An efficient protocol was optimized for shoot regeneration, shoot proliferation, and rooting from *in vivo* buds of yam varieties on MS medium supplemented with specific PGRs. Furthermore, thermo-treatments followed by meristem culture was carried out and YMV-free plantlets were produced from the virus infected *in vitro* plantlets and hardened in the screenhouse.

The explant initiation rate and shoot proliferation showed significant differences among the treatments and the tested varieties. Overall, the MS medium supplemented with intermediate concentrations and combinations of BAP (0.5–1.0 mg/L) and NAA (0.25–0.5 mg/L), enhanced higher shoot regeneration, shoot proliferation, and shoot elongation as compared to MS medium alone (0 mg/L BAP and NAA) or with higher amount of BAP (3.0 mg/L) and NAA (0.5 mg/L) concentrations. Earlier studies on *in vitro* proliferation of yam varieties showed that MS medium supplemented with BAP (0.5 mg/L) + NAA (0.5 mg/L) was the best combination induced 100% shoot regeneration, and proliferation (Birhan et al. 2021). BAP is vital in cell expansion, and NAA is important in cell division (Arya and Husen 2022), so it is fundamental to maintain an optimal BAP and NAA ratio for shoot proliferation and elongation. A higher proportion of cytokinins (BAP) relative to auxins (NAA) promote a higher shoot proliferation that led to reduced shoot length, whilst lesser ratio of BAP displayed a better shoot elongation caused by reduced multiplication among the shoots (Amiri and Mohammadi 2019).

The result also indicated that optimum levels of hormones vary depending on the yam varieties examined. Both lower and higher concentrations of growth regulators negatively affected the shoot initiation and proliferation in all the varieties tested. This is due to the fact that a lower level of hormones may not be sufficient to prompt shoot proliferation, and a higher level of cytokinins might delay cell division, hence the use of well-adjusted growth regulators is recommended for adequate shoot proliferation in short period of time (Salokhe 2021). The morphological and genetic potential of certain plants species was reliant on the explant tissues used and the PGRs in medium, and their endogenous phytohormone concentrations (Ćosić et al. 2015; Phillips and Garda 2019).

On the other hand, RM with IBA and NAA was more effective as compared to MS medium alone for rooting. The highest percentage of rooting, and the longest root length (cm) were recorded in relatively higher level of IBA (1.5 mg/L) and lower level of NAA (0.5 mg/L) combinations. Overall, the medium level of IBA and NAA enhanced better rooting and other related traits in all varieties, thus selected for the thermotherapy experiments. When the level of IBA increased up to 1.5 mg/L, the percentage of rooting increased proportionally, but when the level is above 2 mg/L, inversely the frequency of rooting gradually decreased owing to the endogenous auxin level (Ćosić et al. 2015). Both IBA and NAA were used in combinations to promote rooting, because the use of IBA or NAA alone was not adequate for root formation as described by Arya and Husen (2022). In agreement with previous report, MS medium supplemented with medium concentrations of auxin (IBA) promoted root induction, root number and root length more than either the higher or lower concentrations of auxins (Singh et al. 2009). However, Birhan et al. (2021) reported the highest percentage of rooting and maximum number

of roots with MS medium supplemented with higher concentrations of NAA (2.0 mg/L) combined with higher IBA (1.5 mg/L).

Tissue culture is useful for growing tissues and organs, as well as eliminating plant viruses, and micro-propagating virus-free planting materials (Abraham 2009; Loyola-Vargas et al. 2018; Abrar et al. 2019). Plants that are vegetatively propagated, like yam, are particularly prone to losses brought on by viruses (Maruthi et al. 2019). In this aspect, a thermo-treatment combined with meristem culture was carried out in this study and the result showed significant differences in percentage of YMV elimination efficiency and the survival rate of the plantlets. Unlike, varieties Aw/Ar/001 and Aw/Ar/005 from *D. rotundata* species, variety Aw/Wo/011 from *D. alata* species showed lower virus elimination efficiency in both *in vitro* and screenhouse, indicating that *D. alata* may has more virus resistance gene or it developed systemic immunity than *D. rotundata* (Penet et al. 2016; Bekele and Bekele 2020). Overall, as thermo-treatment increased from 26 ˚C (control) to 40 ˚C, the virus elimination efficiency was also increased in all tested varieties.

Reports showed that meristem culture alone or in combination with chemotherapy, thermotherapy, or cryotherapy, can result in virus-free plants using *in vitro* methods (Wang et al. 2018; Bhat and Rao 2020; Ita et al. 2020; Abide et al. 2022). In thermotherapy, plants are grown at a higher temperature (> 35 °C) under controlled conditions, which inhibits virus movement toward meristematic cells and reduces viral replication, creating a larger virus-negative in infected shoots. All these heat-induced effects may eventually lead to improved virus eradication by meristem culture (Mignouna et al. 2016, Baizan-Edge et al. 2019; Maruthi et al. 2019). Several scientific studies found that nodes that regenerated in tissue culture after thermotherapy tested negative for viral disease on screening (Panattoni et al. 2013; Maruthi et al. 2019), which

agreed to our results. However, the thermo-treated shoots' recorded lower survival rate as compared to the non-thermo treated once. This inconsistency was balanced by the greater proportion of virus-free plantlets gained after thermo-treatment and this is in agreement to report by Ding et al. (2008), and Ita et al. (2020).

In vitro micro-propagating of the thermo-treated meristem, generated virus free plantlets that showed promising level of hardening on transfer to the screenhouse. This shows that the thermo-treatment was not restricted the growth performance of the plantlets both *in vitro* and in screenhouse settings. Re-evaluation of the screenhouse hardened yam varieties with DAS-ELISA, and RT-PCR after four months of acclimatization indicated slightly higher percentage of YMV in most varieties than the one month thermo-treated plantlets. This suggests that YMV was not entirely eradicated but the viral titer was highly reduced to an insignificant concentration at the time of *in vitro* testing.

Previously, different attempts have been made by researchers to eliminate YMV and other viruses from yam plants using meristem tip culture alone or in combination with other treatments, such as chemotherapy, cryotheraphy, and thermotherapy, and successful elimination of the YMV, and other viruses was reported (Loyola-Vargas et al. 2018; Wang et al. 2018; Phillips and Garda 2019). Even though YMV is the widespread potyvirus threats the production of yam in the study areas (Gogile et al. unpublished data), as well as in the globe and affecting germplasm exchange worldwide, (Luo et al. 2022), little work has been made to study YMV elimination using tissue culture combined with other treatments. In this study, heat treatment followed by meristem tip culture was carried out for the elimination of YMV and higher rate of elimination up to 100% was obtained. However, there are many other viruses infecting yam plants that are not considered in this study. Therefore, the meristem tip culture in combination with other treatments should be

considered in the future to enhance the elimination of YMV and other viruses infecting yam plant.

In conclusion, protocols have been developed and applied for *in vitro* proliferation of yams and the production of disease-free planting materials. Meristem tip culture has been used alone or in combination with specialized *in vitro* virus elimination techniques (thermotherapy) to wipe out YMV. ELISA and RT-PCR used in this study can be adapted in virus cleaning schemes on routine basis. The newly developed *in vitro* propagation and virus-elimination protocols are believed to make significant contribution in improving yam production in many countries, particularly in Africa where YMV is a significant constraint.

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Supplementary Table 1. Profile of yam plant varieties maintained at AARC

No	Species	Variety	Total tubers	Collection site	Altitude (masl)[e]	Latitude	Longitude
1	*D. rotundata*	Bulcha[a]	15	HARC[c]	1708	7º06N	38º39'E
2	*D. rotundata*	Aw/Ar/001[b]	50	Gununo	1780	6°55'N	37°38'E
3	*D. rotundata*	Aw/Ar/005[b]	35	Kucha	1340	6°29’N	36°89’E
4	D. *alata*	Aw/Wo/011[b]	40	AARC[d]	1780	6°50’N	37°45’E

[a] Released variety

[b] farmers’ varieties and we used the name ‘variety’ for both in this paper

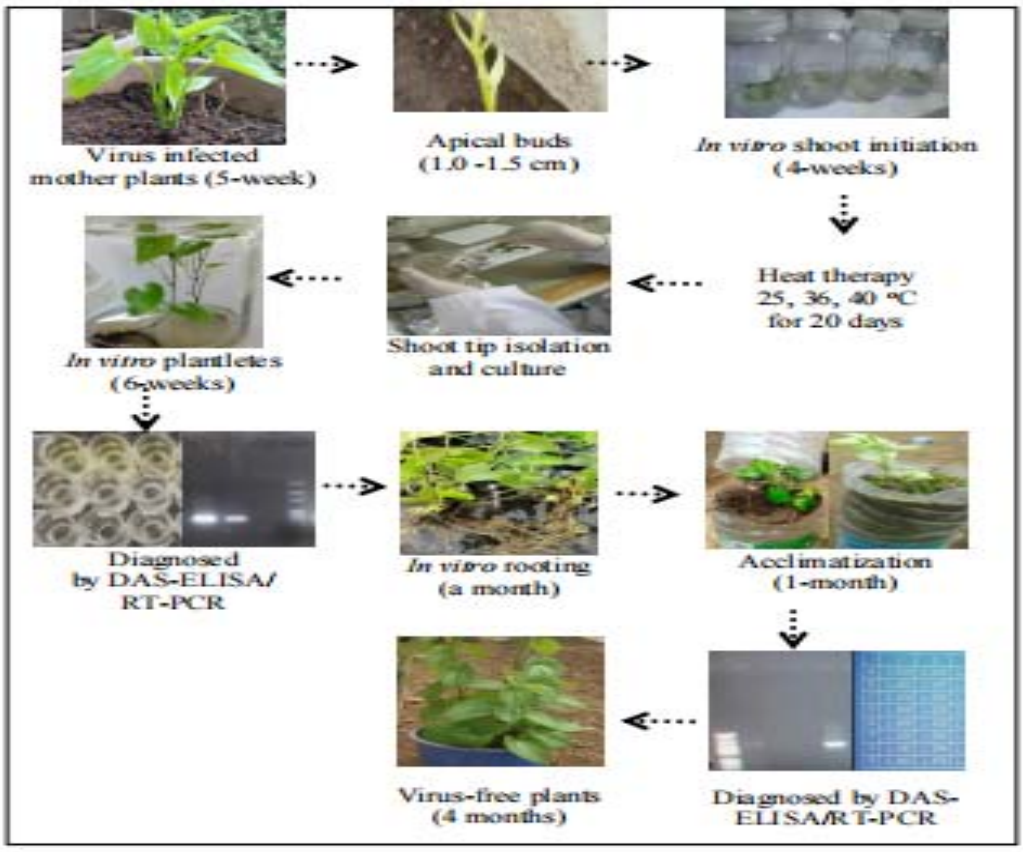
[c] Hawassa agricultural research center

[d] Areka agricultural research center

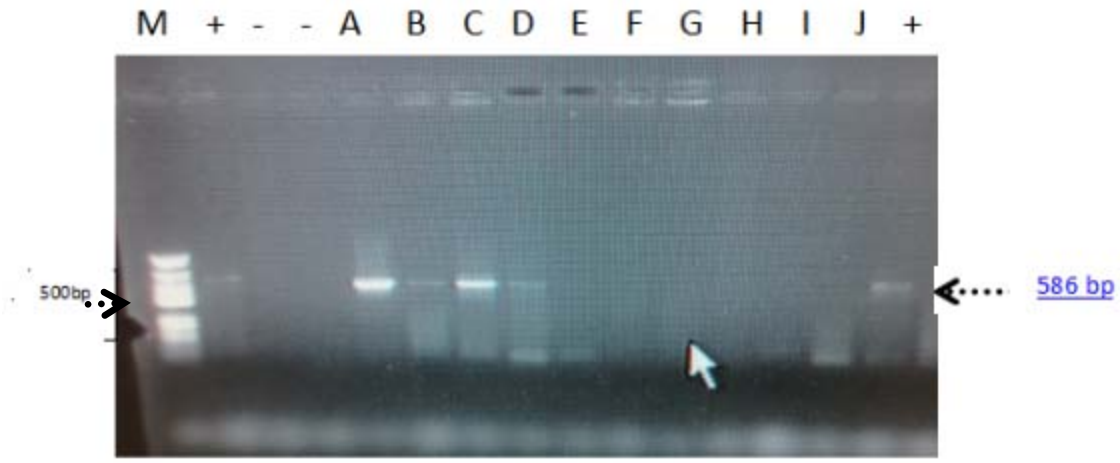
[e] meter above sea level

Supplementary Table 2. Different concentrations and combinations of plant growth hormones for shooting and rooting

Varieties (N)	PGRs (mg/L) for shooting		PGRs (mg/L) for rooting	
	BAP	NAA	IBA	NAA
Bulcha (12)	0.0	0.0	0.0	0.0
		0.25		0.5
		0.5		1.0
	0.5	0.0	0.5	0.0
		0.25		0.25
		0.5		0.5
	1.0	0.0	1.5	0.0
		0.25		0.25
		0.5		0.5
	2.0	0.0	2.0	0.0
		0.25		0.25
		0.5		0.5
	3.0	0.0		
		0.25		
		0.5		
Aw/Ar/001 (12)	0.0	0.0	0.0	0.0
		0.25		0.5
		0.5		1.0
	0.5	0.0	0.5	0.0
		0.25		0.25
		0.5		0.5
	1.0	0.0	1.5	0.0
		0.25		0.25
		0.5		0.5
	2.0	0.0	2.0	0.0
		0.25		0.25
		0.5		0.5
	3.0	0.0		
		0.25		
		0.5		
Aw/Ar/005 (12)	0.0	0.0	0.0	0.0
		0.25		0.5
		0.5		1.0
	0.5	0.0	0.5	0.0
		0.25		0.25
		0.5		0.5
	1.0	0.0	1.5	0.0
		0.25		0.25
		0.5		0.5
	2.0	0.0	2.0	0.0
		0.25		0.25
		0.5		0.5
	3.0	0.0		
		0.25		
		0.5		
Aw/Wo/011(12)	0.0	0.0	0.0	0.0
		0.25		0.5
		0.5		1.0
	0.5	0.0	0.5	0.0
		0.25		0.25
		0.5		0.5
	1.0	0.0	1.5	0.0
		0.25		0.25
		0.5		0.5
	2.0	0.0	2.0	0.0
		0.25		0.25
		0.5		0.5
	3.0	0.0		
		0.25		
		0.5		



Supplementary Fig. 1. Schematic diagram of the production of YMV-free yam plants using thermotherapy and meristem culture.



Supplementary Fig. 2. RT-PCR detection of YMV (586 bp) from YMV-infected plants treated with thermotherapy followed by meristem culture for virus elimination. (M) 100 bp DNA ladder, (+) positive control, (–), negative control (nuclease free water), (A–D) leaf samples at 26 ˚C (control), (E–J) leaf samples thermo-treated at 40 ˚C