The Pharma Innovation Journal 2022; 11(5): 2413-2418

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(5): 2413-2418

www.thepharmajournal.com
Received: 07-01-2022
Accepted: 16-04-2022

Dr. VJ Vasanthi
Associate Professor, Department of Plant Pathology, Pondicherry Institute of Agricultural Sciences, Puducherry, India

Dr. A Vijai Anandh
Associate Professor, Department of Floriculture, JSA College of Agriculture Research and Technology, Avatti-Tittakudi, Cuddalore, Tamil Nadu, India

Dr. P Puvila
Assistant Professor, Department of Agronomy, Don Bosco College of Agriculture, Sagayathottam, Vellore, Tamil Nadu, India

Dr. G Vaidehi
Associate Professor, Department of Horticulture, Bharath Institute of Higher Education and Research, Selaiyur, Chennai, Tamil Nadu, India

Corresponding Author:
Dr. VJ Vasanthi
Associate Professor, Department of Plant Pathology, Pondicherry Institute of Agricultural Sciences, Puducherry, India

Standardization of culture media for meristems and nodal segments in cassava (*Manihot esculenta* Crantz) and virus-indexing using TAS-ELISA

Dr. VJ Vasanthi, Dr. A Vijai Anandh, Dr. P Puvila and Dr. G Vaidehi

Abstract
In this current research, standardization of culture media for meristems, nodal segments and also virus indexing of regenerants using TAS –ELISA were carried out successfully. Five different culture media (Media I for meristems and Media II for nodal segments) were prepared each for *in vitro* propagation of meristems and nodal segments from three different ICMV susceptible cultivars of cassava *viz*., Co-1, Co-3 and H165 along with different concentrations of growth regulators such as Benzyl Amino Purine (BAP), Naphthalene acetic acid (NAA) and Gibberellic acid (GA3). Growth parameters like Nodal length, survival percent, root and shoot induction, leaf and root numbers for each explant were evaluated. Based on analysis of variance ($P< 0.05$), there were significant differences in growth parameters of meristem derived cassava cultivars on Media I combinations. Later nodal segments from regenerants were mass multiplied using Media II with five different combinations of harmones under controlled condition. Cassava cultivar Co-3 meristems cultured in TM2 (Media I composition) 4.4 g/l of MS with BAP (0.25 mg/l), NAA (0.1 mg/l), GA3 (0.03 mg/l) gave highest percent of survival (87%) and highest mean plant height (1.22 cm) and mean root number (0.63) and for *in vitro* nodal segments cultured on N2 (Media II composition) with BAP (0.15 mg/l), NAA(0.02 mg/l), resulted in 99 percent survival. Highest mean plant height (1.32 cm) and mean root number (0.71) were recorded in Co-3 cultivar in nodal explants. Among the different types of soil mixtures tested for hardening, peat and vermiculite (3:1) gave better survival of regenerated plantlets (95 percent). Further virus indexing was carried out for regenerants using TAS- ELISA. This new protocol of (Media I) with TM2 and (Media II) with N2 were standardized for meristem and *in vitro* nodal segments respectively and proved to be promising technology for virus free planting material.

Keywords: NAA, ELISA, ICMV, Meristem, BAP, regeneration, shoot induction, cassava

Introduction
Cassava (*Manihot esculenta Crantz*) is one of the most important starchy tuberized staple food crop widely cultivated in tropics. (Abaca *et al*., 2012) [1] Indian Cassava Mosaic Virus (ICMV) poses serious threat to cassava farmers throughout the world. (Santana *et al*., 2009) [13,] Initial symptoms of the virus are chlorotic speck on young leaves which later tend to enlarge and give rise to a mosaic pattern and then turn yellow in colour. Severe infections lead to reduction in leaf area and in extreme cases leaf gets distorted and becomes like a shoe string (Vasanthi & Shanmugam, 2005, Yona *et al*., 2010, Zainuddin *et al*., 2018) [18, 21, 22].
Cassava is generally propagated through stem cuttings. (Patil *et al*., 2015) [12]. These contribute to the primary mode of spread of Indian cassava mosaic virus (ICMV) and secondary spread is through insect vector white fly (*Bemesia tabaci*). Mosaic disease severely affects tuber yield and quality. To overcome these problems, conventional breeding techniques were tried. They were aimed to achieve ICMV resistant cassava cultivars, but this method was found to be very slow. Hence tissue culture techniques can be used to produce high quality cassava seedlings instead of conventional stem cuttings. From one nodal explant 16000- 17000 plantlets can be produced within a span of one year. This confirmed high range of multiplication *in vitro*. [Shiji *et al*., 2014] [14]. Plant tissue culture technique has been exploited for micro propagation of cassava all over the world. Hence an *in vitro* propagation method such as meristem tip culture was followed to achieve quick and easy supply of disease free cassava planting materials. (Acedo, 2006, Moshkov et *al*., 2008) [2, 9].
Serological assays like enzyme linked immunosorbent assay were used to detect the presence of ICMV in meristem –derived cassava plants (Vasanthi, *et al*., 2001) [19]. TAS-ELISA tests were preferred over conventional methods due to their specificity, speed and the scope they

provide for standardization. In this current study meristem tip culture has been adopted for supply of virus- free cassava planting materials after ELISA tests.

Materials and Methods

Maintenance of Cassava stem cuttings in Glasshouse

Dormant diseased setts of three different cultivars of cassava namely Co-1, Co-3 and H165 (ICMV susceptible cultivars) were cut into sections of two nodes each. The upper part of stem cutting was sealed with paraffin and then the sections were planted in pots in green house at Puducherry. These cassava stem cuttings sprouted within 5 to 7 days. (Acedo, 2006) [2],

Meristem tip culture

Apical meristematic domes of size of 0.5 -0.6 mm were aseptically dissected along with the sprouts, sterilized with 0.1% mercury chloride for 5 minutes and then transferred to sterile water in petri dishes and washed thrice with distilled water. These meristems were then transferred into [Media I with five different hormonal combinations TM1, TM2, TM3, TM4, TM5] test tube measuring 25 x 150 mm containing 15 ml of revised Murashige and Skoog medium (1962) [10] with B5 vitamins in Gamborg medium along with growth regulators Benzyl amino purine [BAP], Naphthalene acetic acid [NAA] and Gibberellic acid [GA3].

Then the tubes were incubated in a growth cabinet programmed to provide a light and dark cycle of 16 /8 hours [3000 lux] and provide fluorescent lamp at 24 ^{0} C Temperature and 75% Relative Humidity. After 30 days, 4- 5 shoots with 4 -5 nodes appeared in inoculated tubes and were again recultured. After 20-25 days, the shoots attained a height of 7 -8 cm with 7-8 nodes. When the shoots were transferred to rooting medium containing MS basal medium with NAA 0.5 mg per litre within 20 – 25 days the shoots produced visible and white roots. (Demeke, *et al*., 2014; Kahn, *et al*., 2012) [7, 8]. For Mass multiplication nodal segments were excised from regenerants and cultured on five different combinations of Media II MS with (N 1, N 2, N 3, N 4, N 5) in test tubes were maintained under controlled conditions.

Hardening of Plants

The regenerated plantlets were removed and placed in test tubes containing sterile distilled water after washing with clean running water. The tubes were incubated at 20-30^{0} C temperature for two days in a culture room and later transferred to empty ice cream cups containing four types of sterile soil mixtures *viz*., Sand: Red Earth: Farm yard manure (FYM) (1:1: 1), Sand: FYM [3:1], peat soil: vermiculate [3:1] and peat soil alone for further transplantation and the performance of plantlets in different soil mixture were studied. The plantlets were incubated in the mist chamber set at 30 -40 ^{0} C during day and 18-25 ^{0}C during night with 70-80 percent RH for 5-7 days. The plantlets in the cups were transferred to mud pots with similar types of potting mixture combinations and grown in green house. (Vasanthi, 1998, Ogero *et al*., 2012, Chauhan *et al*., 2015) [20, 11, 5].

Virus Indexing of meristem-derived cassava plants using TAS-ELISA

Triple Antibody Sandwich ELISA was performed as described by Clark and Adams (1977) [6]. Leaf samples of regenerated plants were used for the purpose and were replicated thrice. Healthy control and disease control and test leaf samples from top, middle and bottom were collected and grounded with buffer at 1:10 and loaded into wells of microtitre plates. Monoclonal antibodies of ACMV SCR 23 and SCR 33 are used as universal standard for detection of ICMV; three monoclonal antibodies *viz*., SCR 17, SCR 58 and SCR 60 for ICMV were obtained from International institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Indian Cassava Mosaic Virus (ICMV) polyclonal antibody was added at the dilution (1:100) to the coating buffer and was incubated for 4 hours at 37 °C. Monoclonal antibody against ICMV was added at the dilution (1: 40). Alkaline phosphatase with p-nitrophenyl phosphate brought yellow colour after incubation and was quickly assessed visually and absorbance was noted in ELISA Reader at 405 nm. ELISA reaction was considered positive when the A405 nm value was greater than the mean plus two standard deviation of untreated control. (Vasanthi *et al*., 2010) [17].

Statistical analysis

All the experiments were carried out based on completely randomized block design (CRBD) under lab conditions. Data were collected on growth parameters such as nodal length, culture survival (two weeks after culture), root growth, leaf formation, plant height, number of roots and leaves per plant (four and six weeks after culture. Analysis of variance (ANOVA) was carried out to determine significant differences among the cultivars and means were separated using Duncan's Multiple Range Test (DMRT). (Ogero *et al*., 2012) [11].

Results

The results of (Table. 1) show the effective preparation of five different harmone combinations both for meristem (Media I) and *in vitro* nodal segments (Media II) in cassava. Hence different types of media consistency such as solid, liquid and semisolid state were tried.

Table 1: Composition of culture media for meristem and nodal segments *in vitro*

S. No		Media composition I (Meristems)		Media composition II (Nodal segments
A.	Common Nutrients	MS 4.40 (g/l), Myo- Inositol 100 (mg/l) Sucrose 30 (g/l) Agar 6 (g/l). pH 5.8		MS 4.40 (g/l), Myo- Inositol 100 (mg/l), Sucrose 30 (g/l) Agar 6 (g/l). pH 5.8
1.	TM1	Solid- Full MS + BAP (0.2 mg/l), NAA (0.1 mg/l), GA3 (0.03 mg/l)	N 1	Solid- Full MS + BAP (0.1 mg/l), NAA (0.02 mg/l)
2.	TM2	Solid- Full MS+ BAP (0.25 mg/l), NAA (0.1 mg/l), GA3 (0.03 mg/l)	N 2	Solid- Full MS+ BAP (0.15 mg/l), NAA (0.02 mg/l) pH 5.8
3.	TM3	Solid -Full MS + BAP (0.15 mg/l), NAA (0.1 mg/l), GA3 (0.05 mg/l)	N 3	Solid- Full MS + BAP (0.25 mg/l), NAA (0.02 mg/l)
4.	TM4	Semisolid- Full MS+ BAP (0.2 mg/ NAA (0.1 mg/l), GA3 (0.03 mg/l)	N 4	Semisolid- Full MS+ BAP (0.2 mg/l), NAA (0.02 mg/l)
5.	TM5	Liquid Full- MS + BAP (0.1 mg/l) NAA (0.1 mg/l), GA3 (0.03 mg/l)	N 5	Liquid -Full MS + BAP (0.1 mg/l), NAA (0.02 mg/l)

*****Common nutrients are for both media I and II except harmonal combinations.

Fig 1: Development of meristem *in vitro*

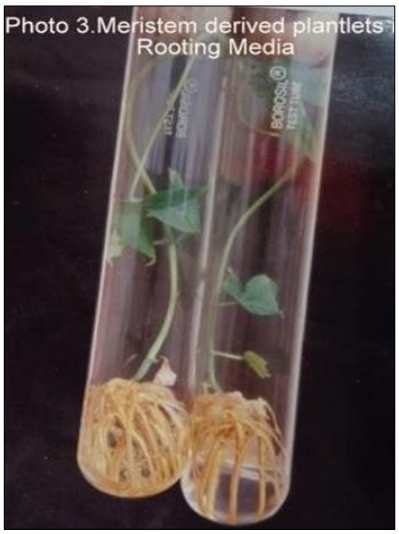

Fig 2: Meristem derived Plantlets

Standardization of different growth harmones in media for Meristems

Six growth parameters like Plant height, survival percent, root and shoot induction, leaf and root number for each explant were evaluated. (Table 2). Based on analysis of variance ($P<$ 0.05) there were significant differences in growth parameters of meristem derived cassava cultivars on Media I with TM2 combination. The media composition was 4.4 g/l of MS with BAP (0.2 mg/l), NAA (0.1 mg/l), GA3 (0.03 mg/l) which gave 87 percent survival of meristem and the highest mean plant height (1.22 cm) and mean root number (0.63) recorded in Co-3. Also the leaf formation and number of leaves per plant were significant (0.41) and (1.57) in Co-3.

Table 2: Effect of media I on meristems of cassava on six growth parameters *in vitro*

Cultiva	Survival	Plant height (cm)	Leaf formation	Number of leaves per plant	Root formation	Number of Roots per plant
Co-1	0.80 ^{b}	1.15 ^{b}	0.36^{a}	1.25^{b}	0.30^{b}	0.54^{b}
Co-3	0.87^{a}	1.22^{a}	0.41^{a}	1.57^{a}	0.61^{a}	0.63^{a}
H 165	0.75^{c}	1.10 ^{c}	0.32^{b}	1.20^{c}	0.26 ^{c}	0.48^{c}

Means with the same letter along the column are not significantly different at 1% level of probability using DMRT

Standardization of different growth hormones in media for Nodal segments

Nodal segments excised from regenerants were cultured in Media II MS + five different combinations of harmones under controlled condition. Aanalysis of variance ($P<$ 0.0,), revealed there were significant differences in growth parameters of meristem derived cassava cultivars on Media I combinations. For nodal explants on MS+ BAP (0.15 mg/l) and NAA (0.02 mg/l) resulted in 99 percent survival and the highest mean plant height (1.32 cm) and mean root number (0.75) were recorded in Co-3 cultivar in N2 media II composition.

Table 3: Effect of media II on *in vitro* nodal segments on cassava growth parameters

Cultivar	Survival	Plant height (cm)	Leaf formation	Number of leaves per plant	Root formation	Number of Roots per plant
Co-1	0.94^{b}	1.21 b	0.54^{a}	1.67 ^{b}	0.10^{b}	0.60^{b}
Co-3	0.99^{a}	1.32^{a}	0.56^{a}	1.98^{a}	0.23^{a}	0.75^{a}
H 165	0.91c	1.14c	0.41b	1.23c	0.08 c	0.55c

Means with the same letter along the column are not significantly different at 1% level of probability using DMRT

Effect of different types of soil mixtures for establishment of *in vitro* grown cassava

In this research, among four different soil mixtures peat: vermiculite (3:1) mixture served best (95 percent) compared to other soil mixture combinations in hardening process of cassava plantlets at mist chamber successfully (Fig.1). Co-3 cultivar performed better in hardening process to this soil mixture compared to other cassava cultivars. Other three mixtures were not suitable for regenerants.

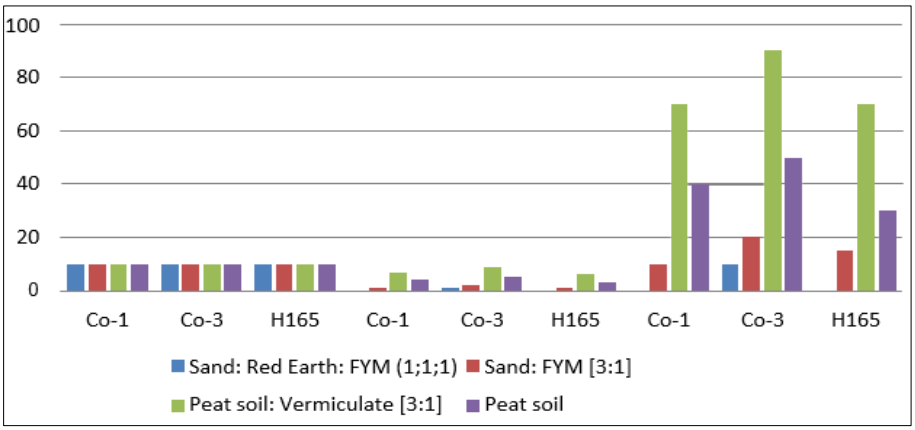

Fig 1: Effect of different Soil Mixtures for hardening of cassava regenerants

Virus-indexing for detection of ICMV in cassava leaves using TAS-ELISA: In the current serological assay, leaf samples from top, middle and bottom of the regenerated plants of three cultivars were collected and tested. The top most leaves were found to be most infected in control and showed higher virus titre under TAS- ELISA tests. The regenerants were free of virus compared to infected control plants based on absorbance values at A 405 nm. (Fig.2).

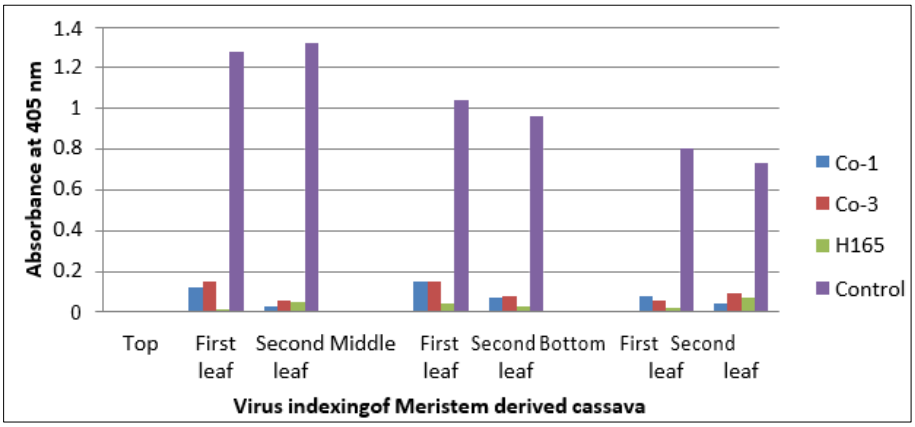

Fig 2: TAS-ELISA for virus-indexing of cassava regenerants

Discussion

Meristem tip culture has been widely associated for production of pathogen free plants and rapid propagation of healthy clones. The presence of viruses in apical meristem cells could be inactive under *in vitro* culture condition. In the present study (Table.1) for standardization of culture media it was very essential to identify suitable combination of MS medium with contrasting hormonal concentrations for meristem growth and mass multiplication of nodal segments *in vitro*. The present study revealed the active role of GA3 which has a stimulating effect on shoot elongation when combined with BAP and NAA (Table.2).

Similar results were reported earlier. Incorporation of GA*3* in the media significantly increased the mean shoot length and full MS supplemented with 40 µM/l GA 3 gave the highest mean shoot length of 8.93 ± 2.67 mm and a protocol was developed for the rapid meristem development and mass propagation of *Manihot esculenta* by Acedo, 2006 [2]. Hence in this study synergistic action of all the three harmones favored the plantlet formation. (Santana *et al*., 2009; Vasanthi *et al*., 2001) [13, 19].

Moreover, in case of liquid medium Media I with TM5 showed better performance initially for meristem development which may be due to increased flow of nutrients. After ten days the liquid cultures degenerated and turned brown. The reason may be excess of water which didn't favor cassava growth. This is in agreement with the findings of Acedo (2006) [2].

Semisolid media (TM4) also contributed better growth with agar as gelling agent. But best shoot and root induction were observed in solid media, especially in TM2 with Media I composition. There was little callus induction which might have induced shoot and root formation. This is in line with certain researchers. (Acedo and Corazon, 2008; Kahn, 20120) [3, 8].

For mass multiplication of meristem-derived virus free cassava planting material, nodal segments were used. Several researchers have suggested for MS with harmone free media or low quantity of harmones is enough for mass multiplication. (Chauhan *et al*., 2018; Acedo and Corazon,

2008; Vasanthi *et al*., 2001) [4, 3, 19]. In this study two different harmones, auxins and cytokinins were used in low quantities for better results. Media II with N2 combination for nodal segments gave better results and proved the necessity of harmones for better survival, growth and shoot and root formation.(Table 3).(Chauhan *et al*.,2015, Moshkov, *et al*., 2008) [3,5,8]

Regarding the effect of different types of soil mixtures for establishment of *in vitro* grown cassava a success rate of 95% was obtained following rooting in peat: vermiculite mixture followed by transfer and acclimatization in a potting medium. The damage to cassava roots during the rinsing and removal of adhered gelling medium were reduced due to vermiculite and peat soil application. *In vitro* rooting of cassava in peat; vermiculite (3:1) increases survival rates during hardening process. (Fig 1). Consecutively, the regenerated cassava shoots produced roots within four weeks in a 0.5 mg/L NAA medium and were successfully acclimatized and transferred to field. (Vasanthi *et al*., 2001, Demeke *et al*., 2014) [19, 7] Hardening process was quite difficult due to poor establishment of plantlets and inadequate surface wax on leaves with an impaired photosynthetic apparatus which was unfavorable for roots and also absence of high sterile conditions. Similar results were reported earlier.

(Chauhan, *et al*. 2018, Vasanthi, 1998) [4, 20]. Shiji *et al*., 2014 [14] reported for shoot initiation, micropropagation and hardening in cassava variety Sree Padmanabha and found that the shoots rooted well *in vitro* and after transferring the plantlets with 4-5 cm length to sterilized vermiculite they found it as suitable base for hardening and subsequently transplanted and got 91 percent success after two months of hardening.

To screen the regenerants for ICMV infection, TAS-ELISA was used in this study. Through virus –indexing symptomless carriers can be quickly detected (Vasanthi, *et al*., 2017 b) [16]. Hence meristem tip culture is an ideal method for ICMV eradication and also established the advent of serological studies in detecting the virus in plant samples. In this study, there were no ICMV infections in meristem- derived cassava plantlets. The virus titre was negligible in meristem- derived plantlets due to lack of infections. Meristem has been selected as it's free from virus because of rapid division of meristem cells compared to the rate of virus multiplication and movement. The mosaic virus is unable to multiply in actively growing apical meristems (Vasanthi *et al*., 2001) [19]. This shows that meristem- derived plants can be a potential source for supply of virus free planting material to farmers.

Conclusion

This research has been focused on standardizing a suitable culture media in developing a better protocol for successful meristem culture and mass multiplication of nodal segments *in vitro*. Further virus-indexing of regenerants were done using TAS-ELISA, so as to assure supply of clean, healthy, virus free planting material to cassava farmers.

Acknowledgement

The first author wishes to thank Dr. V. M. Vasudevan Pillai, CEO, Pillai's Research Institute (PRI), New Panvel, Mumbai. Special Thanks to Dr. C. Ganeche, Chairman, PIAS, Puducherry, Hon. P. Sivanandi, Chennai. M. Ashok, N.S. Sugumar, Dr. B. Arulpari, Dr. B. Kumaran, P. Yuvaraj, NKSMHSS, Vellore. Dr. S. Hemalatha, P. Sasikala, G. Amudha, K. Rukmanidevi, Dr. A. Beaulah, TNAU, for their constant support and encouragement.

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