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Effect of NAA and IBA on *in vitro* regeneration and hardening in cassava (*Manihot esculenta* Crantz.)

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Abstract

As the cassava is a delicate plant to harden during *in vitro* regeneration, huge losses occur during transfer from *in vitro* laboratory to *in-situ* field conditions. One month old *in vitro* plantlets of cassava varieties "Sree Vijaya", "H-226" and "H-165" released by Central Tuber Crops Research Institute, India grown from *in vitro* nodal explants using MS media supplemented with NAA or IBA along with a control were used for hardening studies. For *in vitro* rooting with shoot regeneration, MS media supplemented with 0.3 mgl^{-1} IBA was found to be the best for all the three varieties. Cassava plantlets grown using 0.6% agar in the *in vitro* growth medium with NAA supplement produced least root damage (3%) during washing before hardening. All the varieties grown in MS media supplemented with NAA, showed good results during hardening. Cassava varieties Sree Vijaya and H-226 grown in vermiculite + soil as potting mixture using both Hoagland and fertilizer solution showed maximum success of 87% and 80% respectively. Cassava var. H-165 showed best result from the potting media sand + vermiculite + soil using both Hoagland and fertilizer solution, which gave 100% success. The hardened plantlets could be successfully acclimatized in the green house and transplanted to open field conditions.

Key words: Cassava, Root induction, Shoot regeneration, NAA, IBA, hardening.

Introduction

Cassava is the third-largest source of food carbohydrates in the tropics, after rice and maize (FAO, 2008). As a major staple food crop cultivated in several developing countries, cassava provides a basic diet for about 500 million people. Globally, cassava is grown in an area of 20.73 million ha producing 276.72 million tones of tubers with a productivity of 13.35 t ha^{-1} (FAOSTAT, 2013). In India, it is cultivated in an area of 0.21 million ha with a total production of 7.24 million tones and a productivity of 34.96 t ha^{-1} (FAOSTAT, 2013).

The broad agro-ecological adaptability of cassava, and its ability to produce reasonable yields under unfavourable conditions where most crops cannot, makes it ideal for providing food security at household level and supply cheap and adequate dietary energy. Since cassava is a long duration crop with low, multiplication rate of planting materials (1:10), in traditional propagation, producing planting materials of improved varieties in a short span of time to facilitate adoption by farmers becomes difficult. Past studies indicated that scarcity of quality planting materials affected the spread of improved cassava varieties (Edison et al., 2006; Aladele and Kuta, 2008), besides limiting crop area expansion owing to high cost of seed materials during planting season (Tokula and Ekwe, 2006). Given the vegetative propagation method used for cassava, the infected planting materials transmit the disease causing microorganisms from one generation to anther (Acedo, 2002). Using biotechnological methods and tools for rapid multiplication of improved cassava varieties is one of the strategies to overcome these limitations.

Like other crops, cassava is vulnerable to a broad range of diseases caused by viruses that can cause heavy yield losses. Cassava mosaic disease (CMD) is arguably the most severe virus disease of cassava in India causing an estimated yield loss of 17 to 36% (Malathi, 1985). The CMD produces a variety of foliar symptoms that includes mosaic, mottling and twisted leaflets leading to, an overall reduction in the size of leaves and plants. CMD-affected cassava plants produce few or no tubers depending on the severity of the disease and the age of the plant at the time of infection (Alabi et al., 2011). Many researchers advocated and demonstrated the potential of meristem culture and micropropagation of cassava genotypes to maximize its regeneration and multiplication to produce large number of disease free planting material within a short period. (Kartha et al., 1974; Nair, 1990; Zok et al., 1992; Acedo and Labana, 2008).

One of the major difficulties for the production of planting material in cassava through tissue culture is the hardening stage at which most losses occur. As reported by Jorge (2002), hardening of cassava plants produced *in vitro* is a delicate process that causes huge losses (50 to 95%) (Ospina et al., 2007) and becomes bottleneck in the mass production of cassava planting materials through *in vitro* approaches. The success of the plantlets acclimatization and survival depends on the medium used for regeneration and care with which transplanting is done.There is a need to optimize the plant growth regulators (PGR) concentrations in the MS medium, since they (i) determine the course of morphogenesis in the plant and (ii) PGR requirements vary with species/ genotype (Staden et al., 2008; IITA, 2009). The present study describes an *in vitro* procedure tested for its efficacy for acclimatization of *in vitro* plantlets of cassava during the hardening stage.

Materials and methods

In vitro regeneration of plants

The study was conducted by using apical bud as meristem source from three varieties of cassava- Sree Vijaya, H-165 and H- 226 at Central Tuber Crops Research Institute (CTCRI), Thiruvanathapuram, India. The explants were soaked in soapy solution (one to two drops of teepol in 500 ml water)for fifteen minutes and thoroughly washed with tap water. Then they were dipped in the sterile distilled water and further sterilization was done inside the laminar air flow using 0.1% mercuric chloride ($HgCl_2$) for seven minutes (Shiji et al., 2014) with alternate shaking. After sterilization the tissues were washed five times with sterile distilled water. Apical meristematic domes from the apical buds were dissected and cultured on a Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with benzyl adenine (BA, $5x10^{-7}$ M), naphthalene acetic acid (NAA, 10^{-6} M) and gibberellic acid (GA_3, 10^{-7} M) (Kartha and Gamborg, 1975). The cultures were incubated in culture shelves illuminated with white fluorescent tubes at a room temperature of 25±2°C under 8:16 hours light: dark photoregime.

Micropropagation

After one month of initiation, the *in vitro* regenerated cassava shoots were used as explant source for micropropagation. Nodal segments were prepared and inoculated on to freshly prepared MS basal medium. Subsequent sub culturing was done at one month interval for multiplication.

Root induction with shoot regeneration

The nodal segments excised from *in vitro* shoots of each variety were cultured on MS basal medium supplemented with either á-Naphthalene acetic acid (NAA) or Indole 3-butyric acid (IBA) at varying concentrations (0.1 – 0.5 mg l^{-1}) represented as T_1 to T_{10}along with a control. They were tested for root induction with shoot regeneration in terms of days taken foreach process, percentage of rooting and shooting, number of roots, heights of plantlets and number of nodes. Different agar concentrations (0.6 - 0.8%) were used for media preparation in order to optimize the concentration to produce, least root damage while media washing of the *in vitro* plantlets before hardening.

Hardening

In vitro plantlets grown with the best concentration of NAA and IBA supplemented MS media obtained from our experiments (Table 1, 2 and 3) and basal MS media were selected for hardening. Culture tubes containing plantlets (about 3 to 4 cm height) were taken outside from the growth room and were kept at room temperature for one week. Plantlets which attained 5 to 7 cm height were taken out from the tubes; roots washed carefully

Table 1. Effect of NAA and IBA on root induction with shoot regeneration of *in vitro*nodal segments of cassava (var. Sree Vijaya)

Treatments	Growth regulators (mgl^{-1})		Days to root initiation*	Days to shoot initiation*	Rooting %	Shooting %	Basal callusing	After 3 weeks		
	NAA	IBA						No. of roots*	Height of plantlet*	No. of Nodes*
Control	0	0	5.90±0.28	8.80±1.01	100	90	No	1.80±0.25	3.15±0.37	1.90±0.23
T_1	0.1	0	8.40±0.96	11.10±0.23	90	100	Yes	3.30±0.30	6.00±0.26	2.40±0.16
T_2	0.2	0	9.10±1.05	12.90±0.31	90	100	Yes	2.50±0.45	5.90±0.28	2.40±0.16
T_3	0.3	0	10.90±0.28	10.60±1.78	100	80	Yes	5.10±0.28	5.70±0.98	2.10±0.38
T_5	0.4	0	11.90±0.28	9.00±2.46	100	60	Yes	4.90±0.28	0.80±0.25	0.80±0.25
T_6	0.5	0	11.20±0.25	6.40±2.62	100	30	Yes	6.00±0.26	0.30±0.13	0.00±0.00
T_7	0	0.1	4.70±0.26	8.90±0.31	100	100	No	2.90±0.28	5.00±0.26	2.40±0.16
T_8	0	0.2	4.90±0.28	9.50±0.17	100	100	No	2.70±0.26	4.80±0.25	2.40±0.16
T_9	0	0.3	4.90±0.28	8.80±0.42	100	100	No	3.40±0.16	6.00±0.26	2.50±0.17
T_{10}	0	0.4	5.20±0.25	8.40±1.41	100	80	No	4.20±0.25	5.50±0.95	2.00±0.37
T_{11}	0	0.5	4.90±0.23	8.20±1.80	100	70	No	4.80±0.25	4.70±1.05	1.70±0.40
F value	NA	NA	34.91**	1.4	NA	NA	NA	18.19**	13.18**	10.06**

* Values are expressed as mean ± standard deviation of 10 cultures.

** Significantly different at $p<0.05$.

Table 2. Effect of NAA and IBA on root induction with shoot regeneration of *in vitro* nodal segments of cassava (var. H-165)

Treatments	Growth regulators (mgl^{-1})		Days to root initiation*	Days to shoot initiation*	Rooting %	Shooting %	Basal callusing	After 3 weeks		
	NAA	IBA						No. of roots*	Height of plantlet*	No. of Nodes*
Control	0	0	6.70±0.82	8.10±0.94	90	90	No	1.80±0.33	3.00±0.36	2.00±0.26
T_1	0.1	0	6.10±0.48	10.30±0.73	100	100	Yes	3.40±0.43	5.60±0.27	2.40±0.16
T_2	0.2	0	5.70±0.98	11.80±0.51	80	100	Yes	3.1±0.57	6.70±0.30	2.40±0.16
T_3	0.3	0	8.80±1.49	10.20±1.73	80	80	Yes	3.40±0.96	3.00±0.56	1.80±0.33
T_5	0.4	0	10.40±1.22	8.90±1.96	90	70	Yes	5.20±0.63	3.70±0.83	1.60±0.37
T_6	0.5	0	9.50±1.11	7.60±2.54	90	50	Yes	5.60±0.67	0.70±0.26	0.10±0.10
T_7	0	0.1	4.60±0.31	9.40±0.40	100	100	No	3.30±0.40	4.00±0.21	2.30±0.15
T_8	0	0.2	3.50±0.17	9.50±0.34	100	100	No	3.40±0.52	3.90±0.31	2.60±0.16
T_9	0	0.3	3	10.30±0.47	100	100	No	4.20±0.74	5.80±0.25	2.50±0.17
T_{10}	0	0.4	3.30±0.15	8.10±1.39	100	80	No	4.40±0.64	2.50±0.54	1.40±0.31
T_{11}	0	0.5	3.30±0.15	8.20±1.80	100	70	No	4.20±0.73	2.00±0.47	1.10±0.28
F value	NA	NA	11.33**	0.87	NA	NA	NA	3.18**	16.15**	9.64**

* Values are expressed as mean ± standard deviation of 10 cultures.

** Significantly different at $p<0.05$.

Table 3. Effect of NAA and IBA on root induction with shoot regeneration of *in vitro* nodal segments of cassava (var. H-226)

Treatments	Growth regulators (mgl^{-1})		Days to root initiation*	Days to shoot initiation*	Rooting %	Shooting %	Basal callusing	After 3 weeks		
	NAA	IBA						No. of roots*	Height of plantlet*	No. of Nodes*
Control	0	0	6.70±0.82	8.10±0.94	90	90	No	1.80±0.33	3.00±0.36	2.00±0.26
Control	0	0	6.50±0.76	6.20±0.74	90	90	No	1.30±0.21	2.40±0.31	2.20±0.29
T_1	0.1	0	8.40±0.31	8.60±0.60	100	100	Yes	2.90±0.57	3.30±0.30	1.80±0.25
T_2	0.2	0	8.70±0.37	10.60±0.37	100	100	Yes	4.10±0.48	4.60±0.34	2.60±0.16
T_3	0.3	0	8.30±0.42	8.70±1.47	100	80	No	4.10±0.57	3.60±0.64	1.80±0.33
T_5	0.4	0	9.0±0.3	8.2±1.8	100	70	Yes	4.5±0.5	2.7±0.6	1.3±0.3
T_6	0.5	0	10.90±0.35	8.20±2.24	100	60	Yes	4.80±0.44	1.20±0.36	0.50±0.17
T_7	0	0.1	4.40±0.27	7.10±0.23	100	100	No	3.20±0.42	3.00±0.15	2.10±0.10
T_8	0	0.2	3.90±0.28	8.20±0.29	100	100	No	3.3±0.21	3.00±0.21	2.20±0.13
T_9	0	0.3	4.60±0.85	7.30±0.47	100	100	No	4.10±0.38	3.70±0.26	2.20±0.13
T_{10}	0	0.4	4.10±0.53	8.80±1.53	90	80	Yes	3.70±0.73	2.80±0.61	1.40±0.31
T_{11}	0	0.5	2.70±0.47	8.20±1.81	80	70	Yes	4.00±0.75	2.40±0.58	1.30±0.26
F value	NA	NA	31.01**	0.79	NA	NA	NA	3.59**	4.01**	6.22**

* Values are expressed as mean ± standard deviation of 10 cultures.

** Significantly different at $p<0.05$.

with water to remove traces of growth medium and were transferred to plastic cups containing different sterilized potting mixtures viz., 1.sand+soil (3:1); 2. vermiculite+soil (3:1); 3. sand+vermiculite+soil (1:1:1). 30 ml of Hoagland solution or phosphorous rich fertilizer (Ospina et al., 2002) solution as nutrient supplement, prepared in 0.1% bavistin was poured in the cup and covered with transparent polythene cover to prevent desiccation and to avoid rapid exposure to the environment. The plastic cups with plantlets were placed inside the net house having 35% shade, for acclimatization. One corner of plastic cover was slightly cut opened using scissors after one week and 5 ml of water and Hoagland solution was poured on alternate days using a wash bottle. After two to three weeks, the plastic cover was fully opened and removed and the plantlets were kept in the net house providing the same growing environment up to one month.

One month old plantlets were transplanted to grow bags (7X14 cm) having perforations at the bottom and filled with potting media. The potting medium was prepared by mixing sand, soil and vermiculite in the proportion of 1: 1: 1. After two days, the plantlets were taken out of net house to expose them directly to sunlight for one month. The plantlets were irrigated as per requirement. After two months of hardening (first and second stage), the plantlets were transplanted in the field maintaining adequate management conditions. All statistical analysis was performed using Microsoft Excel Data Analysis Tool Pack (Ver. 2010; Microsoft Corporation).

Result and discussion

Effect of NAA and IBA on root induction and shoot regeneration

Root development of *in vitro* nodal segments of cassava was achieved on solid MS medium supplemented with NAA, or IBA within 3 weeks (Fig. 1 and 2). But 100% root induction with shoot regeneration of cassava varieties Sree Vijaya and H-165, having maximum average numbers of roots (3.40 and 4.20 respectively) were obtained from 0.3 mgl^{-1} IBA supplemented medium (Table 1 and 2). *In vitro* nodal segments showed basal end callusing, followed by emergence of root primordia from the nodal base

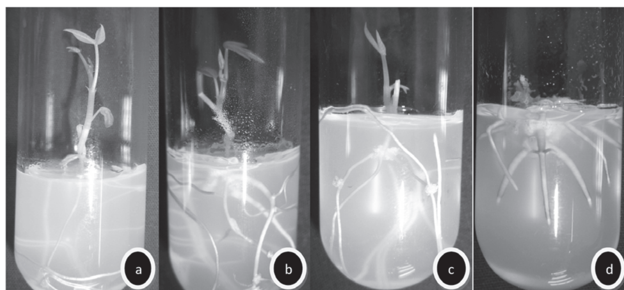

Fig. 1.*In vitro* rooting with shoot regeneration of cassava var. SreeVijaya nodal segments in (a) Control (basal MS medium). (b) Basal MS medium supplemented with 0.1 mgl^{-1} NAA. (c) Basal MS medium supplemented with 0.3 mgl^{-1} IBA. (d) MS basal medium supplemented with 0.5 mgl^{-1} of NAA produce rooting without shoot regeneration.

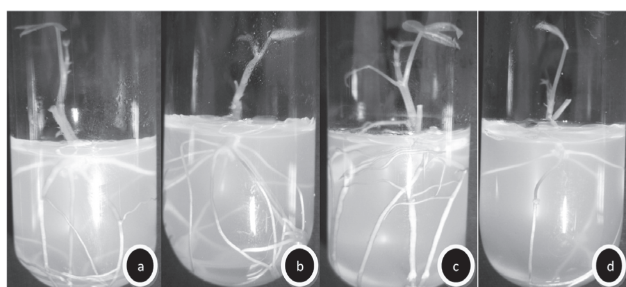

Fig. 2. (a) and (b) *In vitro* rooting with shoot regeneration of cassava var. H-165 nodal segments in basal MS medium supplemented with 0.1 mgl^{-1} NAA and 0.3 mgl^{-1} IBA respectively. (c) and (d) *In vitro* rooting with shoot regeneration of cassava var. H-226 nodal segments in basal MS medium supplemented with 0.2 mgl^{-1} NAA and 0.3 mgl^{-1} IBA respectively.

and rapid root growth in NAA supplemented media. Among the NAA supplemented basal media, best result was obtained from 0.1 mg l^{-1} of NAA which produced 100% root induction with shoot regeneration having 3.40 mean numbers of roots in H-165 (Table 2) and 90% root induction with 100% shoot induction having 3.30 mean numbers of roots in Sree Vijaya (Table 1). In the case of H-226, 100% root induction with shoot regeneration having 4.10 average numbers of roots was obtained from either 0.2 mgl^{-1} of NAA or 0.3 mgl^{-1} of IBA supplemented medium (Table 3). These results indicate that*in vitro* nodal cultures of cassava exhibited differential root and shoot growth differences based on genotypes. Mapayi et al. (2013) also observed similargrowth patterns with three cassava genotypes (92/0326, 95/0289 and I-30572). Less number of roots was recorded from the nodal explant grown in hormone free medium. Analysis of variance (ANOVA) showed significant mean differences for the parameters such as days to root initiation, number of roots, height of plantlets and number of nodes ($p<0.05$).

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama, 1970). A recent African study (Demeke et al. 2014) reported that the regenerated cassava shoots produced an average of 6.14 roots per plant within four weeks in a 0.5 mg l^{-1} NAA medium. An earlier study conducted at ICAR-CTCRI indicated that micropropagated *in vitro* cassava shoots (4-5 cm) transferred to MS medium containing 0.1 mg l^{-1} of NAA produced best results for root induction (Shiji et al., 2014). Another work by Fan et al. (2011) demonstrated that the cytokinin, BAP (0-2.0 mg l^{-1}) was effective on shoot regeneration and the auxin, NAA (0-2.0 mg l^{-1}) proved to be effective for root development in cassava. In NAA supplemented medium, the roots were initiated through the intervention of callus, but in IBA supplemented medium, the resulting plantlets were devoid of callus at the root-shoot junction (Chabukswar and Deodhar, 2005). Moreover, the increased concentration of NAA in the medium proved detrimental to shoot proliferation (Danso et al. 2008).

Acclimatization efficiency of *in vitro* plantlets

Least root damage (3%) was obtained using 0.6% agar in the *in vitro* growth medium with NAA than IBA supplementation during washing of *in vitro* plantlets before hardening (Table 4). The roots of cultures supplemented with NAA were appeared thicker than the control and those which were supplemented with IBA (Fig. 1 and 2). Moreover, higher concentrations of agar caused more root damage (50-83%) during media washing out (Table 4) because, high concentrations of agar result in a harder medium (Beyl, 2000).

Although plantlets having maximum numbers of roots were obtained from MS medium supplemented with IBA, plantlets grown in MS medium supplemented with NAA showed maximum survival percentage. They attained a height of 9-12 cm after one month of transplanting and became ready for the second stage of transplanting (Fig. 6). Plantlets with more than 7 cm height were found unsuitable for hardening due to high, shoot damage while extracting them from culture tubes. Cassava variety H-165 showed best result while hardening in the sterilized

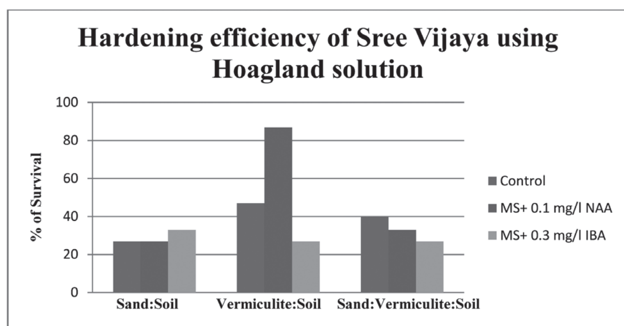

Fig. 3. (a) Effect of different potting mixture with Hoagland solution on survival of *in vitro* plantlets of cassava var. "SreeVijaya".

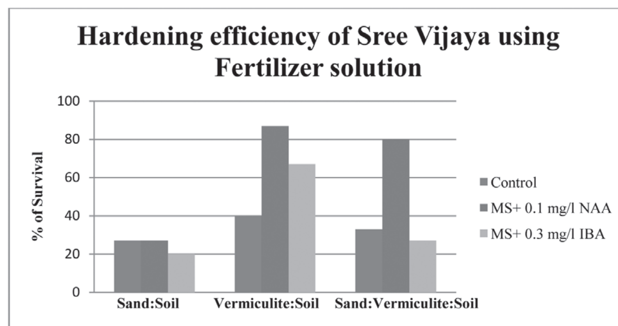

Fig.3. (b) Effect of different potting mixture with fertilizer solution on survival of *in vitro* plantlets of cassava var. "SreeVijaya".

Table 4. Effect of varying concentrations of agar and different growth regulators on root damage during washing of *in vitro* grown casaava plantlets prior to hardening.

Growth medium of *in vitro* plantlets	Conc. of agar (%)	No. of roots	No. of in vitro plantlets washed for hardening	No. of plantlets discarded due to root damage	% of root damage
Control (MS)	0.6	1-3	30	8	27
	0.7	1-3	30	15	50
	0.8	1-3	30	25	83
MS + NAA	0.6	2-4	30	1	3
	0.7	2-4	30	5	17
	0.8	2-4	30	15	50
MS + IBA	0.6	3-5	30	6	20
	0.7	3-5	30	11	37
	0.8	3-5	30	20	67

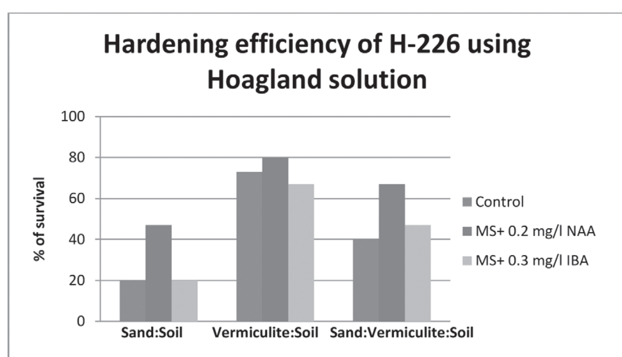

Fig. 4. (a) Effect of different potting mixture with Hoagland solution on survival of *in vitro* plantlets of cassava var. "H-226".

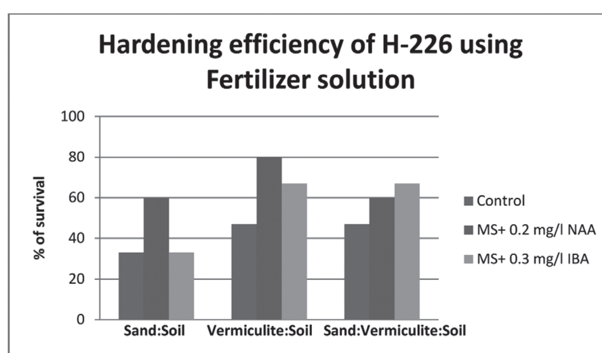

Fig.4. (b) Effect of different potting mixture with fertilizer solution on survival of *in vitro* plantlets of cassava var. "H-226".

potting mixture (sand + vermiculite + soil) using both Hoagland and fertilizer solution as nutrient supplement, which gave 100% success (Fig. 5 and 7). Cassava varieties Sree Vijaya and H-226 grown in vermiculite + soil using both Hoagland and fertilizer solution showed maximum survival of 87% and 80% respectively (Fig. 3, 4 and 7). These results indicate thatthe hardening stage of cassava also exhibit differential acclimatization efficacy based on genotypes.

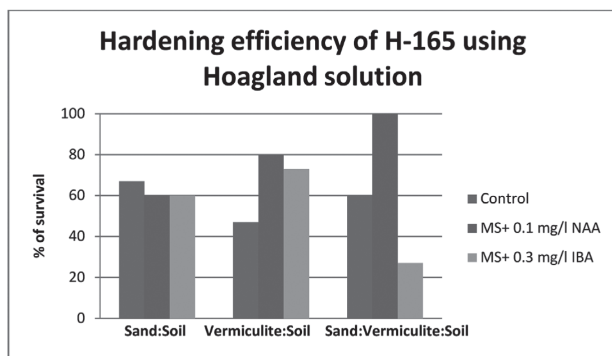

Fig. 5. (a) Effect of different potting mixture with Hoagland solution on survival of *in vitro* plantlets of cassava var. "H-165".

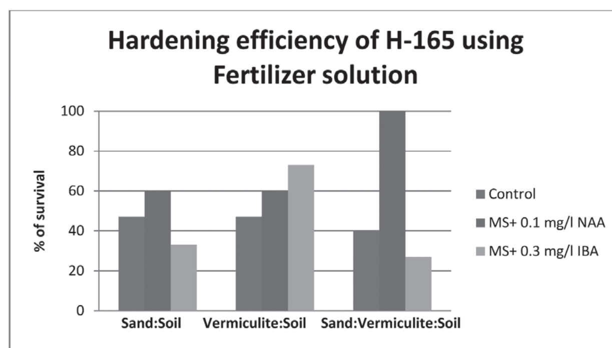

Fig.5. (b) Effect of different potting mixture with fertilizer solution on survival of *in vitro* plantlets of cassava var. "H-165".

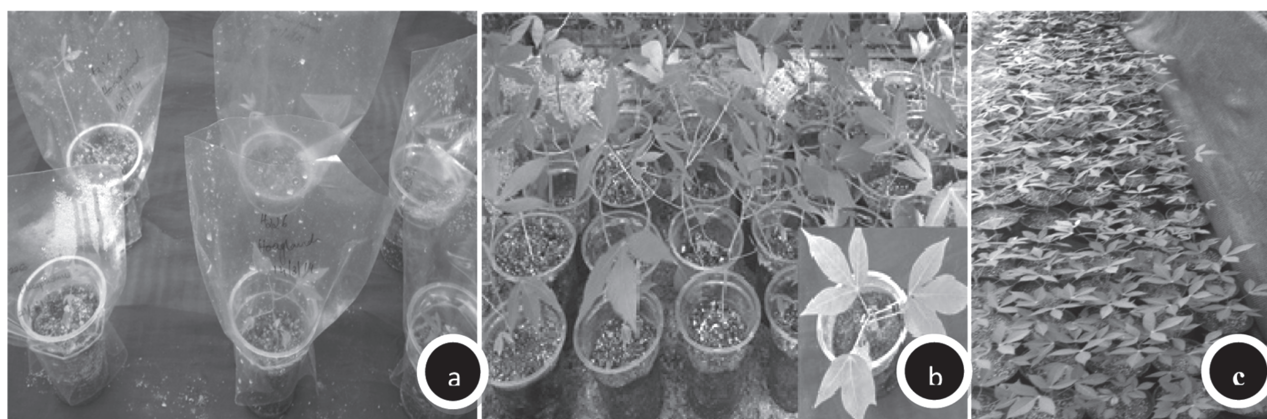

Fig. 6. Different stages of hardening process of *in vitro* regenerated cassava plantlet. (a) *In vitro* regenerated plantlet in the plastic cup covered with polythene bags. (b) After 1 month. (c) After 2 months, the hardened plantlets ready to establish in field.

Fig. 7. *In vitro* plantlets of different cassava varieties after hardening process. (a) Sree Vijaya. (b) H-226. (c) H-165.

Conclusion

Hardening is a crucial phase in micropropagation prior to transplantation of plants to the soil. The *in vitro* plantlets live in 100% relative humidity and they also depend on the medium for the supply of sugar and other nutrients (Ahuja, 1993). During the weaning process, initially the plants were kept under plastic covers for about one week so as to maintain 100% relative humidity. But after one

week the humidity was gradually lowered down by making perforations in the plastic covers. In the meantime the plants developed an efficient root system, built up new leaves and became photosynthetically active. Earlier studies indicated successful acclimatizationof plantlets through different potting media. Ospina et al. (2002) used a sterilized mix of milled and sieved black soil and washed and sieved coarse sand (1:3 ratio) as the substrate and phosphorous rich fertilizer as nutrient for cassava hardening. In an experiment conducted by Demeke et al. (2014) in two cassava varieties, 44/72-NR and 44/72-NW produced 93.3% and 86.7% acclimatized plantlets respectively using sterilized potting mix of forest soil, well decomposed coffee husk and red sand in the ratio 1:1:2. In the present study, MS media supplemented with NAA was found suitable for root induction with shoot regeneration and further hardening process in cassava. *In vitro* plantlets in hardening phase needed extra care and maintenance during washing and transplanting. After two months of hardening the plantlets were transplanted in the field.

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