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Micropropagation for Rapid Multiplication of Planting Material in Cassava (*Manihot esculenta* Crantz)

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Abstract

Rapid multiplication of planting material in cassava through tissue culture techniques such as shoot initiation, micropropagation and hardening was studied in cassava variety Sree Padmanabha. The effect of nodal segments from axillary bud of the field grown cassava cultured in Murashige and Skoog's basal medium supplemented with different concentrations and combinations of 6-Benzylaminopurine (BAP) and Naphthalene acetic acid (NAA) were evaluated based on different parameters viz., number of shoots, number of roots, number of leaves and number of nodes. Minimal risk of contamination was observed with sterilization in 0.1% $HgCl_2$ for 7 min, which was found to be an effective surface sterilant. For *in vitro* regeneration using apical node as explant, MS media with 0.3 mg l^{-1} NAA was found to be the best in terms of shoot having three nodes. When *in vitro* shoots were inoculated in MS media with 0.1 mg l^{-1} NAA, rooting was the most profuse. Nodal explants took one month for development into plantlets having three nodes. Potential number of planting material production from one nodal explant using micropropagation techniques was estimated to be ranging from 16,000 to 17,000 in one year period. Well rooted *in vitro* plantlets in sterilized vermiculite with 4-5 cm length were found suitable for hardening and subsequent transplanting, which gave 91% success after two months of hardening.

Key words: *In vitro* regeneration, micropropagation, BAP, NAA, hardening

Introduction

Cassava (*Manihot esculenta* Crantz.) is an important tropical tuber crop, which is utilized as food, feed and raw material for industrial uses. Cassava is a major staple food crop cultivated in several developing countries, providing the basic diet for around 500 million people. Globally, cassava is grown in an area of 19.64 million ha producing 252.2 million tonnes with a productivity of 12.8 t ha^{-1} (FAO, 2011). In India, the crop is cultivated in an area of 0.2 million ha producing 8.08 million tonnes with a productivity of 36.5 t ha^{-1} (FAO, 2011). Best and Henry (1994) forecasted that cassava production will continue to grow worldwide and therefore it is expected that demands for planting material will increase significantly. Recently, the demand for planting material of cassava has increased drastically due to the need for sources of biofuel also. So, there is a need to develop techniques to rapidly propagate good quality planting material to increase production.

Cassava is usually propagated vegetatively from stem cuttings. Being a long duration crop, the multiplication rate of the planting material is rather slow with a multiplication ratio of approximately 1:10. It also means that the rate of multiplication of new, improved varieties is slow, which delays its adoption. The use of tissue culture based methods for rapid multiplication of improved varieties is one of the viable strategies to solve this limitation. Using *in vitro* culture, multiplication rate can

be improved and planting material can be made available throughout the year.

Cassava is vulnerable to a broad range of diseases caused by viruses. Among them, cassava mosaic disease (CMD) is the most severe and widespread. CMD produces a variety of foliar symptoms that includes mosaic, mottling, misshapen and twisted leaflets, and 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 have reported the possibility of meristem culture and micropropagation of different cassava genotypes for the high frequency regeneration and its multiplication to produce large number of disease free planting material within a short period. (Kartha et al., 1974; Kartha and Gamborg, 1975; Roca, 1979; Nair et al., 1979; Smith et al., 1986; Nair, 1990; Zok et al., 1992; Schopke et al., 1992; Acedo, 2002; Acedo and Labana, 2008). In this context the importance of cassava var. Sree Padmanabha is increasing because it is resistant to cassava mosaic disease. The present study attempts to establish a rapid propagation technique for the year round production of planting material in cassava var. Sree Padmanabha.

Table 1. Effect of surface sterilants on survival of nodal explants from apical buds of cassava var. Sree Padmanabha

Surface sterilant used	Concentration (%)	Period of treatments (min.)	*Survival of explants (%)
Mercuric chloride ($HgCl_2$)	0.08	3	50
	0.08	5	60
	0.08	7	80
	0.1	3	70
	0.1	5	80
	0.1	7	90
Sodium hypochlorite (NaOCl)	0.5	5	10
	0.5	10	20
	0.5	15	20
	1	5	10
	1	10	20
	1	15	30
	2	5	20
	2	10	30
	2	15	30
	3	5	30
	3	10	40
	3	15	40

*Average of ten plants

Materials and Methods

Cassava variety "Sree Padmanabha (MNga-1)" resistant to cassava mosaic disease developed by Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, India, in 2006, was used for the study. First to fourth sub apical nodes of cassava collected from the fields of CTCRI were used as the explant. These nodal explants were surface cleaned with bavistin followed by detergent (teepol). Further sterilization was done inside the laminar air-flow. In order to standardize a suitable sterilization protocol, the explants were treated with surface sterilants like sodium hypochlorite (NaOCl) having different concentrations (0.5 - 3%) for 5, 10 and 15 min and mercuric chloride ($HgCl_2$) having different concentrations (0.08 – 0.1%) for 3, 5 and 7 min (Table 1).

The sterilized nodal explants were placed on basal MS medium (Murashige and Skoog, 1962) supplemented with 2% sucrose (w/v), 0.8% agar agar (w/v) and different concentrations and combinations of plant growth regulators (Table 2). For shoot and root induction, 15 combinations of NAA and BAP in different concentrations such as 0.1 – 0.5 mg l^{-1} and 0.5 – 1.5 mg l^{-1}, respectively designated as T_1 to T_{15} along with a control was tried. The pH of the medium was adjusted to 5.8 using 1N NaOH and 1N HCl before autoclaving. The cultures were maintained at 25±2°C for 8:16 hours light: dark photo regime, using cool-white fluorescent tubes. The effect of the culture media having different combinations of growth regulators on explant regeneration was observed based on the number of days taken for initial shoot and root initiation and best responding explants were used for further propagation. Four replicates were used for each treatment and all the experiments were repeated twice.

In vitro regenerated plantlets were used as explant source for micropropagation. Nodal segments were prepared and inoculated onto freshly prepared MS medium with different concentrations of NAA (T_1 - 0.1, T_2 - 0.3 and T_3 - 0.5 mg l^{-1}) along with a control (Table 3) for rooting.

Table 2. Effect of NAA and BAP on shoot and root initiation from nodal explants of cassava (var. Sree Padmanabha) after 1 month of inoculation

Treatments	Hormonal supplements (mg l^{-1})		Days to initiation	Shoot shoot (cm) for sub culturing	No. of length available	No. nodes leaves	Days to of initiation	No. root of roots
	NAA	BAP						
control	0	0	4-5	3	2	4	5-6	1
T_1	0.1	0	8-9	1.5	1	2	8-9	3-4
T_2	0.3	0	6-8	6	3	4	7-8	6-8
T_3	0.5	0	6-8	6	3	3	7-8	6-8
T_4	0	0.5	8-9	1	-	-	-	-
T_5	0	1	9-10	0.5	-	-	-	-
T_6	0	1.5	11-12	0.5	-	-	-	-
T_7	0.1	0.5	8-9	1.5	-	-	-	-
T_8	0.1	1	-	0	-	-	-	-
T_9	0.1	1.5	9-10	1	-	-	-	-
T_{10}	0.3	0.5	10-11	1	-	-	-	-
T_{11}	0.3	1	9-10	1	-	-	-	-
T_{12}	0.3	1.5	13-15	1	-	-	-	-
T_{13}	0.5	0.5	10-12	2	-	-	-	-
T_{14}	0.5	1	13-15	0.5	-	-	-	-
T_{15}	0.5	1.5	13-15	0.5	-	-	-	-

For hardening, well rooted plantlets having 4-5 cm length were selected. Plantlets were taken out from the test tubes, roots washed, kept on a moist paper and transplanted immediately. Acclimatization of regenerated plantlets was carried out using two types of potting media-sterilized vermiculite and unsterilized vermiculite. The potting media was filled in plastic cups (having perforations at the base), up to half and 15 ml of 0.1% bavistin solution was poured in it. After that, plantlets were placed inside the plastic cups and filled with more vermiculite to keep the plantlets erect without disturbing its roots. Fifteen ml of 0.1% bavistin solution was poured again. The cups were covered with transparent polythene cover having perforations at the top for pouring water and nutrient solution. The plastic cups with plantlets were placed inside the net house, having 35% shade, for acclimatization. After one week, water and Hoagland solution was poured on alternate days using a wash bottle through the holes. After two weeks, the polythene cover was removed and plantlets were kept in the net house for one month.

Table 3. Effect of NAA on root induction of *in vitro* nodal explants of cassava (var. Sree Padmanabha) during micropropagation

Treatments	Concentration of NAA (mg l^{-1})	Percentage of basal end callusing	Days to root initiation	Number of roots
Control	0	-	10-12	1
T_1	0.1	100	6-8	4
T_2	0.3	100	10-11	3
T_3	0.5	100	-	-

One month old plantlets were transplanted to grow bags (7 ×14 cm) containing potting media having perforations at the bottom. The potting media was prepared by mixing sand, soil and vermiculite in the proportion 1: 1: 1. After two days in the net house, the plantlets were kept outside in such a way that it received direct sunlight for one month. Water was sprayed as required on all days. After two months of hardening (first and second stage) the plantlets were planted in the field

maintaining adequate management conditions.

Potential number of planting material that could be produced from one nodal explant per year through micropropagation was calculated as follows:

Time required for the regeneration of cassava nodal explant into *in vitro* plantlets with 3 node = 1 month

Time taken for the subculturing of *in vitro* plantlet at one month interval for multiplication = 9 months

Number of *in vitro* plantlets produced after 9 month of subculturing from one nodal explant = 3^9

Time required for the hardening of *in vitro* plantlets = 2 months

Assumed loss during regeneration and hardening = 16 - 18% of plantlets, the number of planting material available after 1 year was calculated.

Results and Discussion

For surface sterilization, 0.1% $HgCl_2$ for 7 min was found to be effective, since the percentage of survival of explants was found to be higher (90% survival) compared to the other concentrations of $HgCl_2$ (0.08%) tried and other treatments using different concentrations of NaOCl (0.5 - 3 %) (Table 1). Researchers used different sterilization methods for sterilizing cassava nodes *in vitro.* Cacai et al. (2013) sterilized cassava nodes with an initial wash using 70% ethanol for 5 min followed by 10% NaOCl for 20 min. Demeke et al. (2014) followed approximately the same method, but with 0.1% NaOCl and 1-3 drops of Tween-20 for 10 min, after initial soaking in 70% ethyl alcohol for 1 min. Fan et al. (2011) also sterilized the explant with 75% alcohol for 60 sec followed by 0.1% $HgCl_2$ for 5 min.

Nair and Chandra Babu (1996) reported the surface sterilization treatment with $HgCl_2$ (0.1%) for 2 min followed by a dip in 70% ethanol for 2 min as an effective way for nodal culture of lesser yam. Kharat et al. (2008) also reported that 0.1% of $HgCl_2$ for 4 min as an effective surface sterilant for lesser yam, whereas, in greater yam, 0.08% of $HgCl_2$ for 2 min was found to be an effective surface sterilant (Rajitha et al., 2011).

Effect of NAA and BAP on organogenesis

The response of cassava nodal explants cultured on MS media supplemented with growth regulators at different concentrations over a period of one month is presented in Table 2. MS medium with NAA alone produced better results in terms of response of explants to shoot initiation, intermodal elongation/shoot length, number of nodes, number of leaves and number of roots when compared to the other treatments. Within 6–8 days, shoot initiation was observed and further shoot with unopened leaves started emerging in two weeks' time (Fig. 1). Deepthi et al. (2010) reported that shoot regeneration efficiency increased from the first node from the top to the fourth node of the apical bud. In this study also the third and fourth node showed best results than the first and second nodes.

Of the 15 combinations of BAP and NAA tested for *in vitro* regeneration of cassava, T_2 and T_3 elicited optimal response after one month of culture with an average shoot length of 6 cm as compared to the control (Table 2). T_2 and T_3 also resulted in the fastest root initiation within 7-8 days, with an average mean of 6-8 roots per explant after one month of culture. Since the concentration of NAA was lower in T_2 as compared to T_3, the former can be taken as the best treatment for maximum shoot and root regeneration. Strong and long roots helped the plant to survive for longer periods and also helped in greater absorption of nutrients from the medium.

The growth regulator BAP and combinations of BAP and NAA (T_4-T_{15}) had no significant effect on shoot and root regeneration beyond the normal observation period of four weeks. MS medium with different concentrations of BAP showed, broad shoot, short internodes, unopened leaves and little or no callusing at the basal end of explants. Combinations of BAP and NAA, also showed broad stem, short internodes and unopened leaves but with compact callus at the basal end of the explants. Rooting was not observed in both types of treatments (T_4-T_6 and T_7-T_{15}). Because of short internodes, nodes were unavailable for sub culturing after one month of inoculation thereby affecting further multiplication.

The production of plants from axillary buds has proved to be the most applicable and reliable method of *in vitro* propagation. According to George and Sherrington (1984) shoot tip/nodal segment culture depended on stimulating axillary shoot growth by the incorporation of growth regulators into the medium. Fan et al. (2011) reported that the cytokinin, BAP (0-2.0 mg l^{-1}) was effective on shoot regeneration and the auxin, NAA (0-

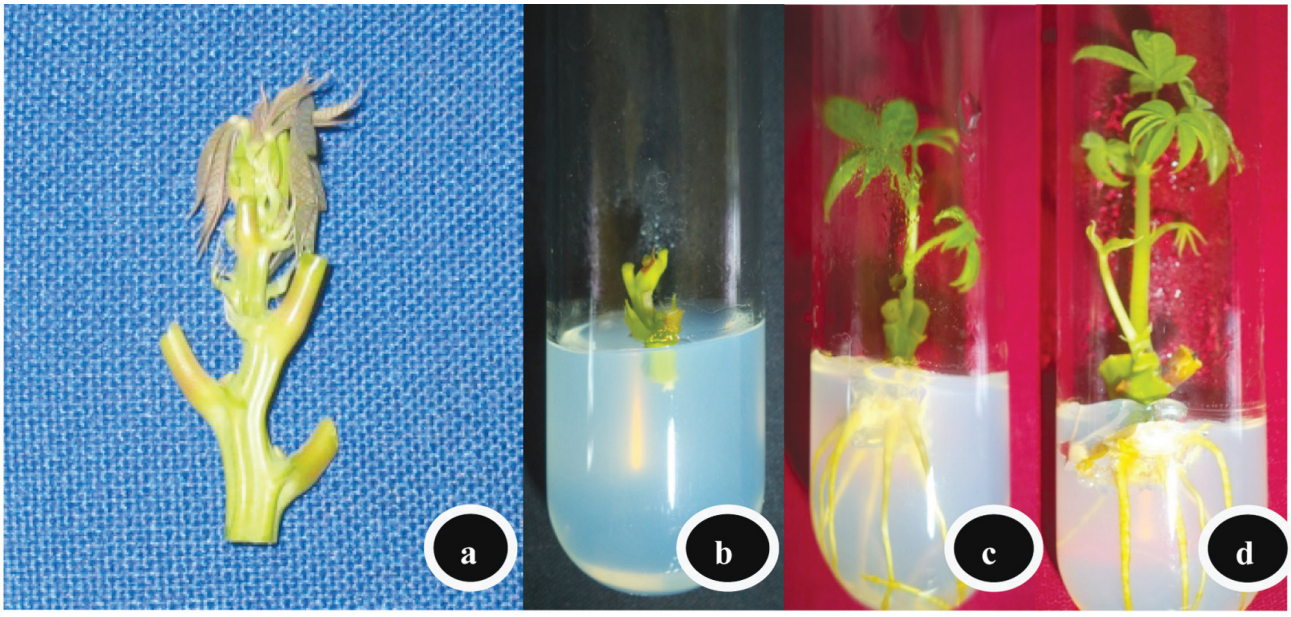

Fig. 1. *In vitro* regeneration of cassava plantlet from the nodal explant. a. Apical portion of field grown cassava used as explant source; b. First day of inoculation; c. After two weeks of inoculation; d. After one month of inoculation

2.0 mg l^{-1}) proved to be effective on root development in cassava. Konan et al. (2006) and Acedo (2009) revealed that shoot multiplication of many crops including cassava could be enhanced with a relatively higher concentration of cytokinins; while rooting is promoted by the use of auxins. Kane (2005) also reported cytokinins, BAP/Kinetin (0.01-10 mg l^{-1}) and auxin, NAA (0.01-10 mg l^{-1}) as the most widely used and effective plant growth regulators for shoot multiplication and root induction, respectively. Several workers succeeded in *in vitro* nodal culture of cassava and its shoot multiplication (Smith et al., 1986; Konan et al., 1997; Konan et al., 2006; Medina et al., 2006). The meristem culture protocol established by Acedo (2002) in 'Golden Yellow' variety of cassava showed shoot and root regeneration in MS media supplemented with combinations of growth regulators such as gibberellic acid (0.25 mg l^{-1}), BAP (0.1 mg l^{-1}) and NAA (0.2 mg l^{-1}).

Many researchers reported the combinations of MS media with NAA and BAP for *in vitro* regeneration in yams (Nair and Chandra Babu, 1996; Acedo, 2003; Kharat et al., 2008). For *in vitro* regeneration in greater yams, MS media with 0.5 mg l^{-1} NAA and 0.75 mg l^{-1} BAP with 0.1% activated charcoal was found to be the best (Rajitha et al., 2011). But according to Acedo (2003) the MS basal media sufficed for stock plant establishment in purple yams.

Induction of rooting from regenerated shoots

Subculturing of *in vitro* regenerated plantlets in MS media supplemented with different concentrations of NAA (0.1-0.3 mg l^{-1}) and control showed shoot initiation within 8-10 days, but T_1 (0.1 mg l^{-1} NAA) alone showed best root formation. Here, basal end callusing, followed by emergence of root primordia was observed from the nodal base within 6 – 8 days after inoculation followed by rapid root growth. Higher concentrations of NAA induced callusing at the basal end of explants. In T_3 (0.3 mg l^{-1}) rooting was not observed. The nodal segments sub cultured in control (MS basal media) showed healthy shoot, but no sufficient roots was formed for hardening (Table 3).

Micropropagated *in vitro* shoots (4-5 cm) established in MS basal media when excised and transferred to MS medium containing 0.1 mg l^{-1} NAA showed better response on root induction. Hence, it was identified as the best treatment for induction of roots in the *in vitro* plantlets prior to a month of hardening. Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama, 1970). Demeke et al. (2014) reported that the regenerated cassava shoots produced an average of 6.14 roots within four weeks in a 0.5 mg l^{-1} NAA. In *D. zingiberensis*, half strength MS with NAA (2.0 mg l^{-1}) was adjudged as the best rooting medium (Chen et al.,

2003). Behera et al. (2010) also obtained similar results in greater yam.

Acclimatization efficiency of *in vitro* plantlets

Well rooted plantlets having 4-5 cm length was found better for hardening and had the maximum success (91%) for establishment in sterilized vermiculite (Fig. 2). Moreover, it attained a length of 8-12 cm after one month of transplanting and became ready for the second stage of transplanting. Plantlets with more than 5 cm length were unsuitable for hardening, because it caused shoot damage during extraction from culture tubes. As reported by Jorge (2002), cassava is a delicate plant to harden, and huge losses occur during transfer from *in vitro* laboratory to *ex vitro* field conditions, which requires care and media optimization. Successful result was not obtained from unsterilized vermiculite used as potting media. The success of the plantlet acclimatization and survival directly depended on the care with which transplanting was done.

Many researchers successfully acclimatized plants through different potting media. Bernardo et al. (2002) used a sterilized mix of milled and sieved black soil and washed and sieved coarse sand (1:3 ratio) as substrate for cassava hardening. Acedo (2003) in an experiment in purple yam (*Dioscorea alata*) produced 85-100% hardened plantlets, using garden soil or mixture of garden soil and river sand as potting medium. Lesser yam (*Dioscorea esculenta* (Lour.) Burk) showed 60-80% survival in the potting media containing soil, sand and cocopeat mixed in a 1:1:1 ratio after one month (Kharat et al., 2008). However, sterilized vermiculite was found to be the best medium for hardening for cassava plantlets in the present study.

Micropropagation: a potential technique for rapid multiplication of planting material

In the traditional system of planting, cassava setts of 20 cm length with 10 to 12 nodes are used as planting material. At this rate, planting material multiplication ratio is 1:10 in one year. In minisett technique, ratio could be enhanced to 1:70 by using two node cuttings (George and Nedunchezhiyan, 2008). But in micropropagation, after initiation of nodal explant, it can produce 3-4 nodal plantlets within one month; which can be sub-cultured at one month interval for 9 months to produce 3^9 ie., 19,683 *in vitro* plantlets. These plantlets required about 2 months for hardening and establishment under *ex vitro* conditions. Here, 16-18% losses were assumed to occur due to contamination and regeneration difficulties during subculturing and hardening stages. Thus through micropropagation technique, number of planting material produced from one nodal explant was estimated to be as high as about 16,000 to 17,000 within one year if the laboratory requirements for *in vitro* propagation are not limiting. Acedo (2002) also obtained 3-4 nodal plantlet from a nodal explant after one month of inoculation in cassava and estimated the potential number of planting material that could be produced from one nodal explant as 159,432 to 3,774,873 after one year, if space and manpower resources were not limiting, assuming a 10% allowance for culture contamination and degeneration. Bernardo et al. (2002) obtained 75,000 plants in one

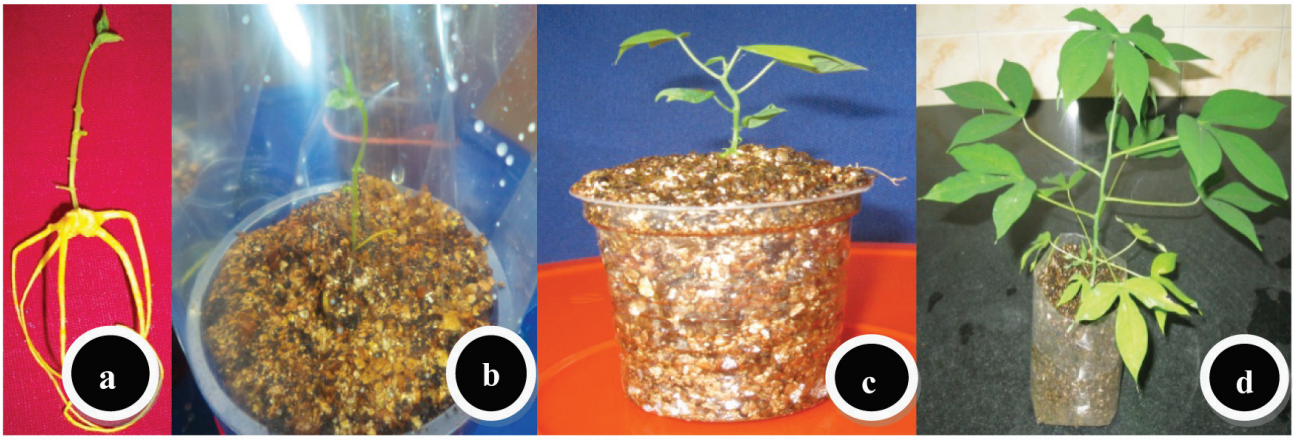

Fig. 2. Different stages of hardening of *in vitro* cassava plantlet. a. Washed *in vitro* plantlet; b. Regenerated plantlets in plastic cups covered with polythene bags; c. After 2 weeks of hardening; d. After 2 months of hardening

year from 10 *in vitro* plantlets of cassava, assuming only 5% losses due to contamination and regeneration. In his experiment, explants took 6 weeks for development into plantlets having 4 nodes. Moreover, from the 36th week of multiplication some plantlets were taken for further multiplication *in vitro* and majority was taken out for hardening.

Conclusion

It can be concluded that MS medium with 0.3 mg l^{-1} NAA was found to be the best for shoot and root regeneration in "Sree Padmanabha" variety of cassava. Micropropagated *in vitro* shoots (4-5 cm) cultured in MS basal media when excised and transferred to MS medium containing 0.1 mg l^{-1} of NAA showed the best response on root induction. Potential number for multiplication of planting material over a period of one year through micropropagation techniques was estimated to range from 16,000 to 17,000. Well rooted, *in vitro* plantlets with 4-5 cm length in sterilized vermiculite used as potting media were found suitable for hardening. After two months of hardening, the plantlets were transplanted in the field. Thus micropropagation offers better scope for rapid multiplication of disease free planting material in cassava.

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