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Conservation of medicinal yam *in vitro*: Effect of ionic strength, sucrose, mannitol, ABA and low temperature

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ABSTRACT

The effects of ionic strength, sucrose, ABA, mannitol and low temperature was investigated on the development of slow growth cultures for *in vitro* conservation of medicinal yam germplasm. Though higher concentration of mannitol (5 %), lower concentration of ionic strength (1/4 MS+ 0.5 % sucrose) and sucrose (0.5 % and 1%) induced significantly high growth reduction in the plantlets, however, these treatments except sucrose not only negatively affected survival of the cultures but also abnormal leaf morphology and multiple shoot formation was observed with increase in concentration of mannitol. Sucrose (0.5 % and 1 %) not only induced slow growth but also reduced the shoot production and showed no effect on survival of cultures even maintained up to 180 days without subculturing. Storage of culture in normal MS media at low temperature (18±1°C) maintained upto 150 days not only reduced the growth parameters but also induced development of both basal and aerial microtubers. Use of low temperature could be the best option for monocrop repositories of yam *in vitro*. However, reduced dose of sucrose can be a suitable and economically sustainable method for *in vitro* conservation of *D. floribunda* at 25±2°C.

Key words: *Dioscorea floribunda, in vitro* conservation, *in vitro* tuberization, low temperature.

INTRODUCTION

Medicinal yam (*Dioscorea floribunda* Mart. & Gal.) is a native of Mexico and Central America. It has been commercially exploited in several countries being one of the most important sources of diosgenin, used in steroidal drugs. The World Health Organization (WHO) has estimated that the present demand for medicinal plants is approximately US $14 billion per year and the demand for medicinal plants is likely to increase more than US $5 trillion in 2050 (Verma *et al*., 18). In view of this demand, over exploitation of this living resource may make the species endangered for which conservation is essentially required. Since *ex vitro* conservation of the genetic resources is not economical, *in vitro* conservation of such plant genetic resources is a common method to conserve this species. Besides, acting as auxiliary measures, it has the potential to overcome some limitations associated with ex *vitro* conservation and to facilitate the exchange of pest free germplasm for which a conservation protocol is essentially required without compromising of the survival of plants, genetic stability and subculture interval. The minimal growth method is the most common *in vitro* culture which directly restricts the growth and development of plantlets by reduction of the plant metabolism and the increase of the subculture time without affecting the tissue or plant viability. This slow growth restriction is achieved by manipulating the environmental conditions (temperature, photoperiod, light intensity etc.) or culture media composition (organic and inorganic nutrients, osmotic regulators, or growth inhibitors) along with incubation period (Engelmann, 7).

With reports to date being meager for development of slow growth shoot cultures for medium-term conservation of this valuable medicinal species of *Dioscorea in vitro*, the present study describes the effect of various slow growth treatments {reduced strength of media composition, reduced doses of sucrose, mannitol and abscicic acid (ABA) in the culture media under normal culture room temperature (25 ± 2°C) and low temperature (18 ± 1°C)} on growth characteristics of *Dioscorea in vitro* so as to develop a suitable and efficient protocol for conservation of the genetic diversity of this medicinal yam.

MATERIALS AND METHODS

This work was carried out at Tissue Culture and Cryopreservation Unit, NBPGR, Pusa Campus, New Delhi. The tubers of *D. floribunda* were obtained originally from NBPGR regional station, Trissur, Kerala. These tubers were planted in pots with 1:1 FYM and soil in the net house. After 2-3 months nodal segments of about 2-3cm long with axillary bud were isolated from net house grown plants. Explants

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were thoroughly washed with tap water and surface sterilized with rectified spirit for 30-60 seconds followed by rinsing with sterile distilled water for 2-3 times. Then the explants were again treated with 0.1% $HgCl_2$ for 15-20 minutes with occasional gentle swirling and washed with sterilized DD water for 3-4 times. The explants were trimmed off to about 1-1.5 cm from the basal portion and inoculated on to basal MS medium (Murasighe and Skoog, 13).

The basal MS medium supplemented with 0.15 mgL^{-1} NAA, 3% sucrose were used for bud proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1 M NaOH or HCl before autoclaving. The MS medium was gelled with 0.8 % (w/v) agar (Qualigen, Bombay, India). Routinely, 25mL of molten media was dispensed into culture tubes (25 × 150 mm), plugged with non-absorbent cotton wrapped in one layer of cheesecloth and sterilized at 121ºC for 15min. All cultures were incubated at 25 ± 2°C under 16-h photoperiod at a light intensity of 42 µmol (photon) m^{-2} s^{-1}. Cultures were subcultured after 2-3 months of *in vitro* establishment on to fresh medium consisting of the same media composition.

In the first experiment, micropropagated plantlets grown in MS media supplemented with different slow growth treatments such as reduced concentrations of ionic strength, different concentration of ABA, mannitol and sucrose (Table 1) were subjected to normal culture room temperature of 25 ± 2°C and were maintained for a period of 180 days. In the second experiment, micropropagated plantlets inoculated in normal MS medium were subjected to grow at low temperature of 18 ± 1°C. Stress analysis for proline and total soluble sugar (TSS) was carried out following the methods of Bates *et al*. (2) and Hedge and Hofreiter (9), respectively for the cultures grown at 18± 1°C and at 25± 2°C after 90 days of storage from the pooled leaf sample collected from five cultures and was replicated thrice.

The experiment was laid out in a completely randomized design with 10 replicates of each treatment and critical difference (C.D.) was analysed at $P \leq 0.05$ level of significance. Periodical observations of the cultures for the measurement of plant height, number of shoots and length of roots at 15 days interval was recorded upto 180 days of storage. In case of low temperature experiment, data analysis for the above growth parameters was done upto 90 days.

RESULTS AND DISCUSSION

In this investigation, normal MS culture media was manipulated by various slow growth treatments *viz.,* reducing dose of sucrose and strength of MS medium, adding up ABA and mannitol to reduce the growth of the cultures for minimizing the frequency of subculturing period and also to enhance the survival of the cultures maintained at normal culture room temperature of 25±2°C. Secondly, the cultures incubated in normal MS media were maintained at low temperature of 18±1°C for a period of 150 days to evaluate its effect on growth parameters of *D. floribunda*. This is the first report in case of *D. floribunda* where various growth treatments were used to induce slow growth in shoot cultures.

Table 1. *In vitro* germplasm conservation treatment of *D. floribunda* at 25±2°C.

Treatment	Strength of MS medium (%)	Sucrose (%)	ABA (mg l^{-1})	Mannitol (%)
T1(control)	100	3.0	0	0
T2-3/4 MS	75	3.0	0	0
T3-1/2MS	50	3.0	0	0
T4-1/4 MS	25	3.0	0	0
T5-1/4 MS+0.5 S	25	0.5	0	0
T6- ABA 2	100	3.0	2.0	0
T7- ABA5	100	3.0	5.0	0
T8- ABA 10	100	3.0	10.0	0
T9- Mannitol 1.0	100	3.0	0	1.0
T10-Mannitol 1.5	100	3.0	0	1.5
T11-Mannitol 3.0	100	3.0	0	3.0
T12-Mannitol 5.0	100	3.0	0	5.0
T13-Sucrose 2	100	2.0	0	0
T14-Sucrose 1.5	100	1.5	0	0
T15-Sucrose 1.0	100	1.0	0	0
T16-Sucrose 0.5	100	0.5	0	0

The treatments at all concentrations for slow growth were evaluated under 25±2°C differentially reduced the growth parameters and this reduction was observed from initial stage and persisted up to 180 days (Table 1). Considering the individual treatments, it was observed that reduction of the strength of MS media retarded maximum shoot (3.82 cm) and root growth (2.04 cm) in T5 (1/4 MS medium+ 0.5 % Sucrose) followed by T4 (1/4 MS +3 % sucrose) after 180 days of culture (Table 2; Fig. 1A). Similar result was reported in slow growth conservation of *E. chlorocorymbos* (Lopez-Puc,10). This supports the view that on reducing the mineral salt concentration, cultures in general grow slowly due to availability of lesser nutrient and low water uptake (Shibli *et al*., 16). However, in this study two treatments like T5 and T4 affected 60% and 80%

Table 2. Effect of *in vitro* conservation culture condition on minimal growth of *D. floribunda* plantlets after 180 days of conservation.

Treatment	Shoot length (cm)	Number of Shoot	Root length (cm)	Survival (%)
T1(control)	8.85 [1]	11.65 [1]	4.37 [1]	100
T2-3/4 MS	7.49 [3]	9.29 [3]	3.45 [10]	100
T3-1/2MS	6.38 [6]	9.06 [4]	3.04 [11]	100
T4-1/4 MS	5.15 [10]	8.20 [6]	2.71 [12]	80
T5-1/4 MS+0.5 S	3.82 [15]	5.48 [9]	2.04 [16]	60
T6- ABA 2	6.60 [5]	5.17 [10]	4.23 [2]	100
T7- ABA5	5.34 [9]	3.55 [15]	2.34 [14]	100
T8- ABA 10	4.71 [11]	3.47 [16]	2.33 [15]	60
T9- Mannitol 1.0	7.11 [4]	7.70 [7]	4.11 [3]	100
T10-Mannitol 1.5	5.99 [7]	8.31 [5]	4.08 [4]	100
T11-Mannitol 3.0	4.67 [12]	10.58 [2]	3.83 [7]	60
T12-Mannitol 5.0	3.18 [16]	7.55 [8]	2.63 [13]	40
T13-Sucrose 2	7.76 [2]	4.46 [11]	3.96 [5]	100
T14-Sucrose 1.5	5.57 [8]	4.38 [12]	3.92 [6]	100
T15-Sucrose 1.0	4.56 [13]	4.27 [13]	3.55 [9]	100
T16-Sucrose 0.5	4.26 [14]	3.94 [14]	3.56 [8]	100
C.D(0.05)	0.205	0.177	0.190	
S.E(d)	0.104	0.090	0.096	

Note: Number in column followed by number in superscript are the rank of the treatment.

survival of the cultures. Moreover, the growth of the plantlets was found to be poor and simultaneously the colour of the leaves turned into pale green.

The abscisic acid (ABA) commonly known as plant growth suppressor generally acts as an endogenous growth retardant and has been used for growth reduction of *in vitro* cultures. A linear tendency of significant decrease in shoot length was observed with the increase of the ABA concentrations (2, 5 and 10 mg L-1) (Table 2; Fig. 1B) after 180 days of culture. However, maximum shoot length reduction was observed with the treatment T8 (10 mg L^{-1}). This inhibitory effect of ABA can be attributed to limited cell extensibility and inhibition of cell division cycle (Finkelstein *et al.*, 8). Shoot production was significantly reduced in the treatments T7 (ABA 5mg L^{-1}) and T8 (ABA 10 mg $L^{-1)}$ compared to other slow growth adjuvenants used in this study. However, 60 % survival was observed in the culture supplemented with higher dose of ABA 10mgL^{-1}, which is in agreement with results of Silva and Scherwinski-Pereira (17) in *P. aduncum* cultures.

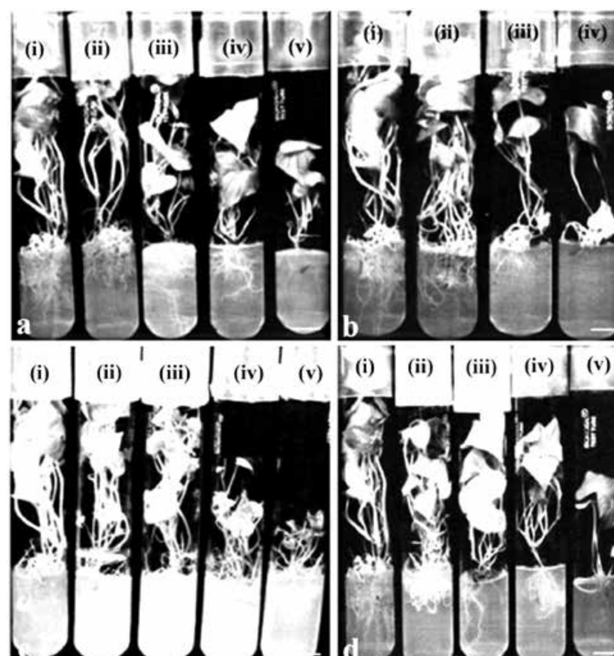

Fig. 1. Effect of slow growth treatments on growth parameters: **A,** Effect of reduced media constituents on shoot growth (i) control (ii) 3/4 MS +3% sucrose (iii) 1/2MS+3% sucrose (iv) 1/4 MS +3% sucrose (v) 1/4 MS +0.5 % sucrose; **B,** Effect of ABA on shoot growth (i) control (ii) ABA 2mgL^{-1}(iii) ABA 5mgL^{-1}(iv) ABA10mgL^{-1}; **C**,Effect of mannitol on shoot growth (i) control (ii) mannitol 1% (iii) mannitol 1.5% (iv) mannitol 3.0%(v) mannitol 5.0 %; **D,** Effect of reduced dose of sucrose on shoot growth(i) control (ii) sucrose 2% (iii) sucrose 1.5% (iv)sucrose 1.0% (v) sucrose 0.5%.

All the concentration of mannitol (10,15, 30 and 50 g L^{-1}) used in this study significantly reduced the shoot length, root length and number of shoot after 180 days of culture (Table 2; Fig. 1C). Decline in growth parameters were found significantly high at mannitol concentration of 5 % followed by 3 %. This growth retardation is generally due to reduction in mineral uptake by cell through differences in osmotic pressures which strongly inhibits the plant cell, tissue and organ growth mainly by impairing the gain of photoassimilates e.g. by inducing stomata closure or lowering the activity of photosynthetic enzymes. However, the treatments T12 and T11 affected 40 % and 60 % survival of the cultures, respectively. Both higher and lower concentration of mannitol (<3% and ≥3%) was found to have negative effect on survival of microshoots (Lopez-Puc, 10; Munoz *et al.*, 12). This envisages that the lethal concentrations of mannitol seem to be species specific and in other way storage time can influence significantly the survival of the cultures (Bekhett and Usama, 3). Culture

abnormalities like formation of small and miniature leaves along with formation of multiple shoot with dark green glassy leaves were observed in the cultures employing mannitol at higher concentrations (3 and 5%) at the initial stage which failed to expand even after 180 days of storage (Fig.1C). This seems to be undesirable as difficulties may be encountered in the recovery of normal plantlets from such phenotype. Formation of such distorted leaves may be due to long time exposure of plantlets to high osmotic potential which could damage the physiology of some tissues or organs as observed in *Habanero pepper* (Montalvo-Peniche *et al*., 11).Also mannitol concentration (1.0,1.5 and 3%) showed ascending trend in generation of shoot numbers of 7.70, 8.31 and 10.58 respectively. However, less number of shoots (7.55) were produced at enhanced concentration of 5 % mannitol. Changes in endogenous growth regulators such as ABA and ethylene under this osomotic stress conditions may be responsible for increase in shoot number (Voesenek and Vanderveen, 19) where as reduction in shoot number at the higher concentration of mannitol may be due to its lethality.

Sucrose, the most commonly used carbohydrate, is universally used as energy source in plant micropropagation. Reduced concentration of sucrose (2.0, 1.5, 1.0 and 0.5 %) as against normally used 3 % in MS media had not only profound effect in inducing slow growth in the plantlets maintained at 25 ±2°C, but also all the cultures were found with normal growth and morphology upto 180 days of conservation without sub-culturing (Fig. 1D). Maximum growth retardation (mean shoot length of 4.26 cm) along with drastic reduction in number of shoots (3.94) was observed with the treatment (T16; 0.5% sucrose) followed by the treatment T15 (1.0 % sucrose). Similar result was reported in oil palm for growth reduction of microplants (Camillo and Scherwinski-Pereira, 6). Poor response in shoot length at low concentration of sucrose may be due to the competition for nutrient and osmotic components. Moreover, cleavage of sucrose at low concentration does not produce NADH leading to relatively low respiration efficiency and growth rate (Bianco and Rieger, 4).

Of all the applied treatments at 25±2°C, extreme or higher concentration of different treatments though significantly reduced the growth parameters but had a negative effect on survival of the cultures. However, treatments at lower concentration such as T2 (3/4MS + 3% sucrose),T3 (1/2 MS+ 3% sucrose), T6 (ABA- 2 mg L^{-1}),T7(ABA-5 mg L-1), T9 (mannitol-1%) and T10 (mannitol - 1.5%) reduced the shoot growth without affecting survival of the cultures; the growth reduction was much more pronounced in case of lower concentration of sucrose (0.5% and 1%) which not only had beneficial effect on survival but also highly effective in limiting the microshoot height upto 180 days of culture without subculturing. The plantlets showed normal morphology along with normal growth.

In vitro conservation at low temperature was reported in yam species of *Dioscorea alata* and *D. rotundata* (Ng and Ng, 14) at 18 - 22°C. The reduction of incubation temperature prolonged the subculturing cycle by reducing the growth rate (Engelmann, 7). Lower root and shoot growth of 2.46 cm and 4.14 cm respectively was found in the cultures maintained at 18±1°C (Table 3:Fig. 2A) as compared to the cultures maintained at 25± 2°C after three months of storage. At this low temperature, number of shoot produced (3.30) was also significantly less as compared to control (4.14).

Formation of both basal and aerial tubers was found in the cultures maintained at 18 ±1°C after 150 days of storage (Fig. 2B). On an average one tuber was formed in each culture and most of the cultures also found to bear aerial tubers developed from the nodal position on the shoots above the medium. Fine roots were also found to develop from the microtubers and it was also observed that from the swollen basal tubers new shoots also started proliferating which has the advantage of maintaining the germplasm for long period. This study provides the first information on both aerial and basal microtuber formation in *D.floribunda* at 18 ±1°C, though, it was reported that the culture of this medicinal yam maintained at 23-25°C resulted in formation of only aerial tubers (Sengupta *et al*., 15).

Table 3. Effect of low temperature on *D. floribunda* plantlets and accumulation of proline and total soluble sugar after 90 days of conservation.

Temperature (°C)	Shoot length (cm)	Number of shoot	Root length (cm)	Proline (g/g of fresh weight)	TSS (µg/g of fresh weight)
25±2°C	6.88	4.14	3.50	0.01	18.00
18±1°C	4.14	3.30	2.46	0.09	272.57
C.D(5%)	0.141	0.103	0.077	0.03	6.25
S.E(d)	0.070	0.051	0.039	0.007	1.453

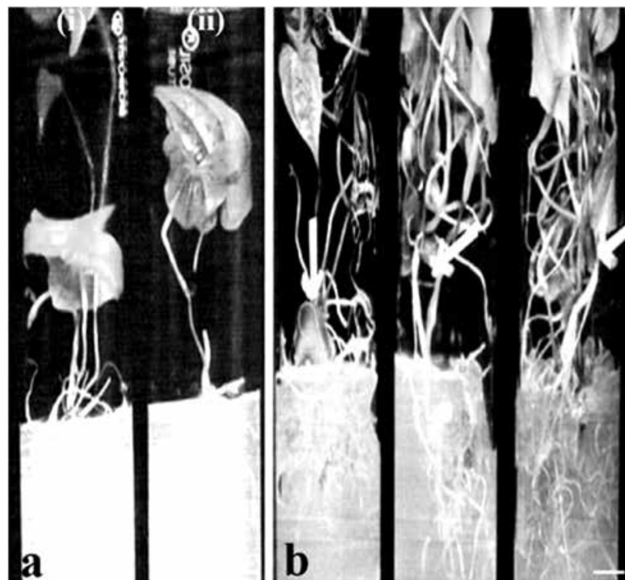

Fig. 2. Effect of low temperature on growth parameters: **A,** Effect of temperture on shoot growth (i) at control (25±2°C) (ii) at 18±1°C; **B,** Effect of low temperature (18±1°C) on tuberisation.

It was interestingly found that cultures maintained at low temperature (18 ±1°C) accumulated more proline (0.09 g/g) and total soluble sugar (TSS-272.33 μg/g) than the cultures maintained at 25± 2°C. Increase in proline and total soluble sugar content at low temperature of 4°C was also reported in rapeseed shoots grown *in vitro* (Burbulis *et al*., 5). Formation of microtuber in the plantlets grown at this low temperature may be correlated to increase in TSS as high dose of sugar was also reported to induce microtubers in other species of *Dioscorea* (Balogun *et al*., 1). However there is no report available on effect of proline on inducing microtubers *in vitro*. Reduction in growth parameters and formation of microtubers at low temperature not only increases the storage period but also allows obtaining propagating material with physiological quality and acts as suitable material for international exchange of germplasm.

A superior treatment for maintenance of germplasm *in vitro* is one that results in uniform growth reduction, stable over time and with minimal distortion in plant morphology. Based on these criteria, the use of low temperature (18±1°C) may be the best option in those *in vitro* repositories of yam in which single crop is supposed to be conserved besides at 25±2°C, sucrose at lower concentration could be a suitable and economically sustainable method for *in vitro* conservation of *D. floribunda* as it showed beneficial effect on survival of the plantlets and effective in limiting the microshoot height upto 180 days of culture. Additionally, plantlets were observed with normal morphology along with good and pronouncing growth which may foster favourable microplant growth. It remains to be determined what would be the genetic integrity of the culture after prolonged storage on sucrose in future. Also this study needs further standardisation of optimum concentration of sucrose and the storage duration for which safe and long term storage can be possible without frequent subculturing and without hampering genetic integrity.

REFERENCES

1. Balogun, M.O. Fawole, I. Ng, S,Y,C. Ng, N.Q. Shiwachi, H. and Kikuno, H. 2006. Interactions among cultural factors in microtuberization of white yam (*Dioscorea rotundata*). Trop. Sci. **46**: 55-59.
2. Bates, L. S. Waldren, R. P. and Teare, I.D.1973. Rapid determination of free proline for water stress studies. Plant Soil, **39**: 205-07.
3. Bekhett, S. and Usama, I.A. 2007. *In vitro* conservation of globe artichoke (*Cynara scolymus* L.) Int. J. Agric. Biol. **9**: 404-07.
4. Bianco, LR. and Rieger, M. 2002. Roles of sorbitol and sucrose in growth and respiration of encore peaches at the three developmental stages. J. American Soc. Hort. Sci. **127**: 297-302.
5. Burbulis, N. Jonytiene, V. Kupriene, R. and Blinstrubiene, A. 2011. Changes in proline and soluble sugars content during cold acclimation of winter rapeseed shoots in vitro. J. Food Agric. Environ. **9**: 371-74.
6. Camillo, J. and Scherwinski-Pereira, J.E.S. 2015. *In vitro* maintenance, under slow-growth conditions of oil palm germplasm obtained by embryo rescue Pesq. Agropec. Bras. **50**: 426-29.
7. Engelmann, F. 1991. *In vitro* conservation of tropical plant germplasm. Euphytica, **57**: 227–43.
8. Finkelstein, R.R. Gampala, S.S.L. and Rock, C.D. 2002. Abscisic Acid Signaling in Seeds and Seedlings. Plant Cell, **14**: 15-45.
9. Hedge, J.E. and Hofreiter, B.T. 1962. (Eds) Whistler, R.L. and Bemiller J.N. Carbohydrate chemistry. Academic press, New York. 420 p.
10. Lopez-Puc, G. 2013. An effective *in vitro* slow growth protocol for conservation of the orchid

Epidendrum chlorocorymbus Schltr. Trop. Subtrop. Agro Ecosyst. **16**: 61-68.

11. Montalvo-Peniche, M.C. Mijangos-Cortes, J.O. Iglesias-Andreu, L.G. Nahuat-Dzib, S.L. Barahon a-erez, F. Canto-Flick, A. and Santana-Buzzy, N. 2007. *In Vitro* Germplasm Conservation of Habanero Pepper (*Capsicum chinense* Jacq.) Hort. Sci. **42**: 1247–52.

12. Munoz, M., Oscar, D. Waleska, R. Annelore, W. and Roberto, Q. 2019. Slow growth *in vitro* culture for Conservation of Chilotanum potato germplasm. Chil. J. Agric. Res. **79**: 26-35.

13. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 473–97.

14. Ng, S.YC. and Ng, N.Q. 1997. Germplasm conservation in food yams (*Dioscorea* spp): Constraints, Application and Future prospects. (Eds) Razdan, M.K. and Cocking, E.C. Conservation of plant Genetic resources *in vitro*. Science publishers Inc. U.S.A. pp. 257-86.

15. Sengupta, J. Mitra, G.C. and Sharma, A.K. 1984. Organogenesis and tuberization in cultures of *Dioscorea floribunda.* Plant Cell Tiss. Org. Cult. **3**: 325-31.

16. Shibli, R.A, Shatnawi, M.A., Subaih, W., Ajlouni, M.M. 2006. *In vitro* conservation and cryoconservation of plant genetic resources: a review. World J. Agric. Sci. **2**: 372-82.

17. Silva, T.L. and Scherwinski-Pereira, J.E.S. 2011. *In vitro* conservation of *Piper aduncum* and *Piper hispidinervum* under slowgrowth conditions pesqui. Agropecu. Bras. **46**: 384-89.

18. Verma, P., Mathur, A.K., Jain, S.P., Mathur, A. 2012. *In Vitro* Conservation of Twenty-Three over exploited Medicinal Plants Belonging to the Indian Sub Continent. The Scientific World J. 1-10.

19. Voesenek L.A.C.J, Van der Veen R. 1994. Review: the role of phytohormones in plant stress: too much or too little water. Acta Bot. Neerl. **43**: 91-127

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