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The role of supporting substrates in ex vitro acclimatization and growth of tissue cultured cassava plantlets

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Abstract

Tissue-cultured cassava plantlets were acclimatized on three different supporting substrates (agar matrix, river sand/sawdust and peat pellet/vermiculite) to determine their effect on growth and development of the plantlets. The *in vitro*-raised cassava roots were carefully washed with distilled water before planting with care onto sterile substrates in a humidity tent. To lower the relative humidity in the tent, holes were created on the sides of the tent starting from the third day to the fifth day in a stepwise fashion. Gradual increase in growth were observed after transfer to humidity tent for 2 weeks followed by accelerated growth between 4 to 6 weeks with values (13.22, 22.42 cm, 12.93 and 24.96 cm) for number of leaves and shoot heights on the supporting substrates (river sand/sawdust and peat pellet/vermiculite), respectively, compared to 10.43 (number of leaves) and 16.35 cm (shoot heights) produced on agar matrix. The most significant feature was the development of a dense mass of roots in both river sand/sawdust and peat pellet/vermiculite substrates suggesting that the porosity of these supporting substrates enhanced rooting and growth of these plantlets. The significance of the supporting substrates during acclimatization and their effects on the plantlets survival as well as the overall plant performance and environmental conditions during acclimatization of the TMS 98/0505 cassava variety are also discussed.

Keywords: Agar matrix; *in vitro* cassava plantlets; porosity; peat pellet/vermiculite; rooting; river sand; sawdust.
Abbreviations: AMHT_agar matrix in humidity tent; DMRT_Duncan multiple range test; PPVHT_peat pellet/vermiculite in humidity tent; RS/SDHT_river sand/sawdust in humidity tent.

Introduction

Substrates porosity is inextricably linked to increase in the hydraulic conductivity of plantlet roots. Abnormalities in *in vitro* raised plantlets can be improved significantly by modifying the humidity and the light irradiance in humidity tents *ex vitro*. Under photoautotrophic conditions, establishment of high relative humidity in the humidity tent is very important for the heterotrophic plantlets survival *ex vitro*. In addition to humidity, CO_2, light and supporting substrates are factors that directly affect growth and photosynthetic capacity of plantlets (Kozai, 2010; Norikane et al., 2010; Ubalua and Okoroafor, 2013).

Root hairs constitute a major plant/substrate interface as they represent as much as 70% of the plant root surface (Peterson and Fraquhar, 1996; Parker et al., 2000). Non-functionality of root hairs can drastically reduce water and mineral nutrient uptake, thus representing a limiting key step to acclimatization. Poor survival rate during plantlets acclimatization has been linked to non functionality of the *in vitro* developed rooting system (Gonclaves et al., 1998). Research has shown that *in vitro* grown plantlets exhibit a low capacity for inorganic carbon assimilation because of their heterotrophic metabolism (Premkumar et al., 2001). In addition, the use of air-tight tubes in order to prevent contamination decreases air turbulence and limits inflow of CO_2 in tissue culture tubes. Pospišilova et al., (1999) contends that culture conditions also have very high air humidity and low irradiance, and that the culture media are supplemented with sucrose as carbon and energy sources. During *in vitro* propagation, relative humidity is usually greater than 95%. Moreover, development of optimum waxy cuticle and functional stomata may not be efficient compared with those of the *ex vitro* plants (Seelye et al., 2003; Ubalua and Okoroafor, 2013). Acclimatization of fragile *in vitro* plantlets overcomes these abnormalities by the gradual reduction of the relative humidity in the humidity tent (Bolar et al., 1998; Ubalua and Okoroafor, 2013). This approach leads to improved plantlets transpiration and the development of functional stomata to regulate plant water loss (Seelye et al., 2003).

Sucrose mediated mechanism have been proposed to control down-regulation of photosynthesis by sink (Franck et al., 2006). The low rate of regeneration of the carboxylation substrate RuBP due to the accumulation of soluble sugar in the leaves possibly results in inhibition of photosynthesis (Azcón-Bieto, 1983). Similarly, the poor ventilation of test tubes results in a high relative humidity inside them, which forces *in vitro* plantlets to keep their stomata open (Fabbri et al., 1986), even though malformations in stomata morphology affects aperture and closing, resulting in high stomata conductance (Sáez et al., 2012) suggesting that excessive water loss could contribute to high mortality of plantlets during transfer to *ex vitro* conditions.

The transfer and acclimatization to the *ex vitro* environment is the final but frequently most critical hazardous step in a successful *in vitro* propagation system (Preece and Sutter, 1991). After transfer from test tubes to *ex vitro* environment, these juvenile plantlets require adaptation to their new environment. The nature of supporting substrates and the intrinsic quality of tissue culture-raised plantlets play an important role in the acclimatization process. It has been

reported by various authors that the physical and chemical nature of supporting substrates appreciably affects rooting and re-establishment of plantlets (Kataoka, 1994; Ubalua and Okoroafor, 2013). Moreover, plantlets with well developed root system *in vitro* determine to a large extent their survival during acclimatization.

Cassava (*Manihot esculenta* Crantz) remains the major food crop for consumption in Nigeria. The plant is usually cultivated through vegetative propagation, but cassava's vegetative propagation is a mixed blessing (Thro et al., 1999). The lignified stem cuttings used for planting can survive in the soil in spite of delayed rains, even though seedlings may die. The build-up of systemic infections, especially of viruses and common bacterial blight in planting material are a disadvantage to this method (Thro et al, 1997; Thro et al., 1999). New cassava varieties, by contrast (through tissue culture) are disease-free and often high yielding. In addition, tissue culture technique is independent of the time of the year, and of the amount of the crop in the field and also could be a factor to moderate price swings (Thro et al., 1999). In this study therefore, we investigated three different supporting substrates for *ex vitro* acclimatization. The roles of these supporting substrates on *ex vitro* establishment of these plantlets are discussed.

Results and Discussion

Micro propagated plantlets are generally grown under low light intensity (1,200-3,000 lux) and temperature (25±2^{0}C), hence direct transfer to broad spectrum sunlight (4,000-12,000 lux) and temperature (26-36^{0}C) might cause charring of leaves and wilting of plantlets (Chandra et al., 2010). Lab to field transfer stage of *in vitro* developed plantlets is a critical stage. During this period, extreme care is taken in order to protect the juvenile plantlets from the harsh *ex vitro* environmental conditions.

Acclimatization

It is necessary to adapt the plantlets to the natural conditions by a process of acclimatization. In this study, the *in vitro* raised plantlets were kept in a humidity tent under a shade for 6 weeks under diffused natural light to help them adjust to the conditions of the new environment. This approach helped in partial hardening of the plantlets and enhanced shoot elongation (Lavanya et al., 2009). Prior to acclimatization, the plantlets height were 6.0 cm on average, with at least 4-5 leaves and 10-11 roots (Fig. 1A-D). Acclimatization was on three supporting substrates; agar matrix, peat pellet/vermiculite and river sand/sawdust. The establishment of high relative humidity in the humidity tent mimics heterotrophic conditions that previously existed in the culture room which is necessary for stabilization and plantlets survival *ex vitro* except that the type and concentration of available nutrients and irradiance which was suitable for photosynthesis were now different in the humidity tent (Lesar et al., 2012; Ubalua and Okoroafor, 2013). This practice improved the functioning of the plantlets stomata and the formation and hardening of wax cuticle on the leaves and stems (Ubalua and Okoroafor, 2013).

Transplanting

Generally, *in vitro*-raised plantlets normally follow slow growth pattern during the first 3-6 days of acclimatization (van Huylenbroeck and Debergh, 1996; Estrada-luna et al., 2001; Ubalua and Okorafor, 2013). Similar pattern was observed following transplanting and acclimatization, as depicted by the growth parameters considered within the first 2 weeks of acclimatization (Tables 1, 2 & 3). Sustained growth within the first 4 days suggests that the roots formed during rooting stage *in vitro* (Fig. 1C & D) remained functional during acclimatization. During rooting stage, cuticular transpiration rates gradually decreased because stomatal regulations of water loss are more effective and cuticle and epicuticular wax developes (Fila et al., 1998). This physiological adjustment experienced during acclimatization enhanced the ability of the plantlets to survive transplanting shock and re-establishment (Estrada-Luna *et al*., 2001; Ubalua & Okoroafor, 2013).

Growth and Development

As the relative humidity was gradually reduced on the third day, recovery of the plantlets from water stress was observed, followed by re-emergence of new leaves within 7 days, triggering a switch from photomixotrophic to photoautotrophic mode of nutrition. Leaves developed *in vitro* had low net photosynthetic activity. New leaves produced during and after acclimatization had increased net photosynthesis, particularly when transferred from humidity tent to higher natural light condition that were prevailing under the shade (Estrada-Luna et al., 2001; Ubalua & Okoroafor, 2013). Overall, the plant growth parameters had significant increases after the first 2 weeks of acclimatization (Tables 1, 2 & 3) which is a demonstration of enhanced photoautotrophic competence of the leafy cassava plantlets (Fig. 2A, B & C), the prevailing optimum irradiance and CO_2 conditions in the natural environment of the shade under which the plantlets were acclimatized.

Substrate porosity

The nature of the supporting substrates and the intrinsic quality of the micro propagated plantlets are some of the determining factors controlling the percentage of survival of the *in vitro* plantlets during acclimatization (Afreen-Zobayed et al., 1999). River sand/sawdust and peat pellet/vermiculite are porous substrates. Acclimatization and growth of *in vitro*-raised plantlets is highly correlated with substrates porosity. We observed that the development of roots in the substrates differed in the three substrates (Fig. 2D, E & F). Afreen-Zobayed et al., (1999) reported that the porosity of agar after gelling should be near zero. Labrousse et al., (2012) also contended that roots developed in agar have shorter hairs, larger rhizodermal cells, and less organized root caps than those growing in paper pulp. Roots of the TMS 98/0505 plantlets acclimated in our agar matrix have similar phenotype to hairless root mutants as reported by Parker et al., (2000). The diffusion of O_2 in the agar matrix is slower than in the peat pellet/vermiculite and the river sand/sawdust substrates. Decreased O_2 level in the supporting substrates could be directly associated with a decrease in root-hair length and to a complementary extent with a decrease in root

Table 1: Mean shoot height produced by the *in vitro* cassava (TMS 98/0505) during acclimatization

	Mean shoot height (cm)		
Treatment	2 weeks	4 weeks	6 weeks
AMHT	8.03[b]	11.98[b]	16.35[c]
RS/SDHT	8.69[a]	15.71[a]	22.42[b]
PPVHT	8.37[b]	16.02[a]	24.96[a]

Mean in a column with the same superscript letter do not differ significantly according to DMRT at 5% level of probability.

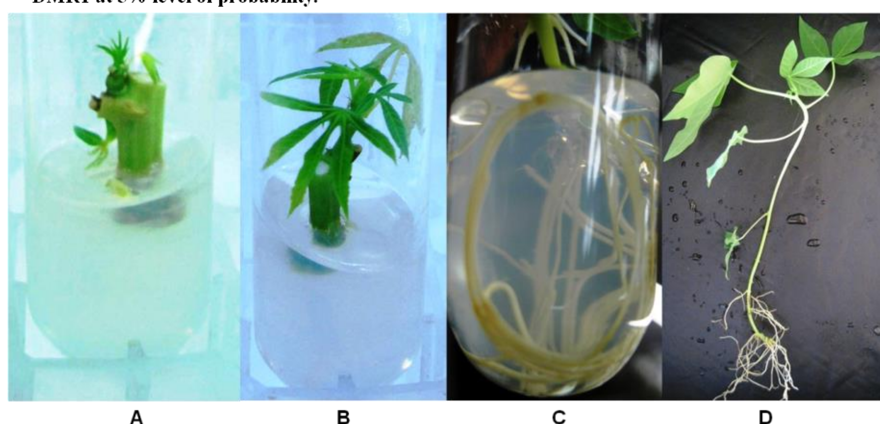

Fig. 1: *In vitro* regeneration of cassava (TMS 98/0505) plantlets.
A: *In vitro* cultured cassava (TMS 98/0505) cutting; B: Growing TMS 98/0505 plantlet; C: Fully developed plantlet roots after 8 weeks in culture; D: Roots freed from agar medium and ready to be transplanted into supporting substrates for acclimatization.

Table 2: Mean number of leaves produced by the *in vitro* cassava (TMS 98/0505) during acclimatization

	Mean number of leaves		
Treatment	2 weeks	4 weeks	6 weeks
AMHT	5.0[b]	8.36[ab]	10.43[ab]
RS/SDHT	6.15[a]	10.15[a]	13.22[a]
PPVHT	6.0[a]	9.65[a]	12.93[a]

Mean in a column with the same superscript letter (s) do not differ significantly according to DMRT at 5% level of probability.

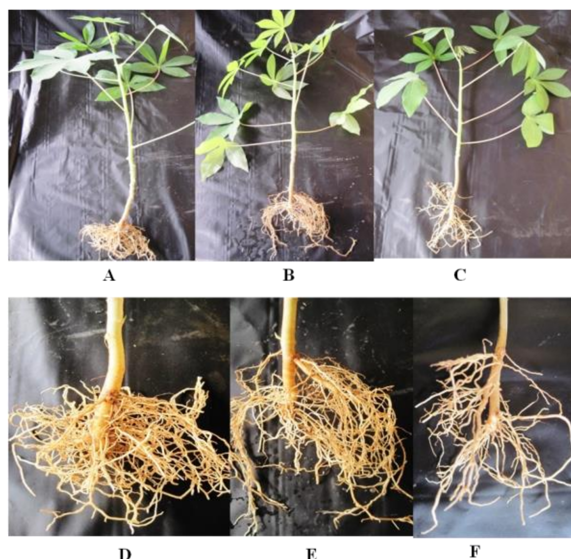

Fig. 2: *In vitro* cassava plantlets acclimatized *ex vitro* in humidity tent for 6 weeks in different types of supporting substrates. Above – From left to right: A; plantlet acclimatized in peat pellet/vermiculite, B; plantlet acclimatized in river sand/sawdust, & C; plantlet acclimatized in agar matrix,
Below – From left to right: D; roots developed in peat pellet/vermiculite, E; roots developed in river sand/sawdust & F; roots developed in agar matrix.

Table 3: Mean number of nodes produced by the *in vitro* cassava (TMS 98/0505) during acclimatization.

	Mean number of nodes		
Treatment	**2 weeks**	**4 weeks**	**6 weeks**
AM/HT	5.0^{b}	8.44^{b}	10.23^{ab}
RS/SDHT	6.15^{a}	10.07^{a}	13.22^{a}
PPVHT	6.0^{a}	9.65^{a}	12.93^{a}

Mean in a column with the same superscript letter (s) do not differ significantly according to DMRT at 5% level of probability.

Fig. 3: Acclimatized cassava plantlets

respiration (Bidel et al., 2000). Among the supporting substrates used in the experiment, porosity was higher in PPVHT and RS/SDHT. PPVHT and RS/SDHT were observed to have profound effects on the growth and development of the cassava plantlets (Tables 1, 2 & 3). The plantlets acclimatized on PPVHT and RS/SDHT produced the highest shoot height of 24.96 and 22.42 cm after 6 weeks (Table 1) respectively. Although the number of leaves and nodes developed were not significant in the two substrates (Table 2 & 3), shoot height was slightly significant (Table 1). However, a significant difference was observed in the pattern of root development (Fig. 2D, E & F). In PPVHT and RS/SDHT substrates, root growth and development were dense and this could be as a result of higher nutrient/water absorption capacity of the roots. Our results revealed that the plantlets acclimatized in PPVHT and RS/SDHT substrates performed far better with morphologically superior roots and shoot systems compared to those in the agar matrix (Fig. 2D, E & F). These observations are in close conformity with the findings of Afreen-Zobayed et al., (1999), Kirdmanche et al., (1995) and Roche et al., (1996) who suggested that fibrous materials such as florialite, vermiculite, ceramic wool, and cellulose plugs are better supporting substrates compared to other gelling agents. Expectedly, agar matrix produced a comparatively low shoot height (16.35 cm), smaller number of leaves (10.43) and nodes (8.44) (Tables 1, 2 & 3). In summary, the obtained results suggest that the quality of the *in vitro*-raised plantlets and the porous nature of the supporting substrates were the main contributor to the complete re-establishment and the turgidity of the acclimatized plantlets as shown in Fig. 2D, E & F. probably by increasing their water/mineral uptake rate after transplanting. The acclimatized plantlets were potted in top soil (Fig. 3) and watered once a day under a shade for 4 weeks of hardening before they were transferred to the field.

Materials and Methods

The study was carried out between June 1 and August 31, 2015.

Plant material

The *in vitro* cassava plantlets (TMS 98/0505) were obtained from the National Root Crops Research Institute (NRCRI) Umudike, Umuahia, Nigeria. They were regenerated and multiplied for a period of 2 months.

Substrate composition

Sawdust is a pulverized by-product or waste product of wood produced by cutting or drilling of woods with a saw or grinder respectively. It is available in any carpenters workshop. The combinations of the substrates were in the ratio of 1:1 for river sand/sawdust and peat pellet/vermiculite. They were properly mixed with water and sterilized by autoclaving at 121^0C for 20 min. Agar matrix is a sterilized agar.

Treatment

The *in vitro*-raised cassava roots (TMS 98/0505) were gently washed with distilled water to remove adhering medium on the roots before planting with care onto sterile substrates: agar matrix (in open test tubes) (AMHT); (river sand/saw dust (RS/SDHT (1:1)), and peat pellet/vermiculite (PPVHT) (1:1) (conventional) in a 5x5 cm transparent polybags. The humidity tent, the transparent polybags containing the supporting substrates, the agar matrix in open test tubes and the plantlets were sprayed with distilled water. The airtight white transparent polythene humidity tent was kept under a shade to reduce water loss and irradiance during the acclimatization period. Starting from day 3, the tent was opened in a stepwise fashion. On the morning of the third day, the relative humidity in the tent was gradually lowered by opening 3 holes of about 1 cm in diameter on the sides of the humidity chamber. On the fourth day, the relative humidity was further lowered by opening a window on the lower side of the tent. A wash bottle containing six grains of fertilizer and water were mixed properly before adequate spraying of the plantlets and the humidity tent as well. The plantlets and the humidity tent were again sprayed with water toward evening time before closing the window. On the morning of the fifth day, the previous window was re-opened and a second window opened on the opposite side of the tent. The plantlets and the humidity tent were again generously sprayed with water.

Traits measured

After 2, 4 and 6 weeks, the following parameters were determined: shoot heights, number of leaves, nodes and root development before transplanting to soil. The plants were watered once a day and occasionally with dilute fertilizer and kept under a shade for another 4 weeks before transplanting to the field.

Statistical Analysis

Each treatment was repeated three times and data recorded 2, 4 and 6 weeks after the beginning of the treatments. Analysis was done using DMRT test at 5% level of significance.

Conclusion

In summary, we concluded that the growth and development of the plantlets were influenced by the quality of the supporting substrates, their physical nature and their nutrient composition. It is therefore suggestive that the porosity of the substrates played a crucial role in the overall development of the plantlets as a result of the enhanced nutrients and water absorption capacity of the roots in the river sand/saw and peat pellet/vermiculite substrates.

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