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Acclimatization of virus-free tissue culture plants of cassava through semi autotropic hydroponics technique

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Abstract

Manihot esculenta Crantz, commonly known as cassava, belongs to the Euphorbiaceae family, and is a crucial tuber crop that serves as a staple food for millions of people worldwide. The acclimatization of cassava tissue culture plantlets faces challenges due to temperature, humidity and microfauna which leads to high mortality rate. To overcome these limitations, semi-autotrophic hydroponics (SAH) emerges as a promising technique for enhancing the large-scale production of cassava plants free from CMD and refining the process of acclimating *in vitro* plants to external conditions. This study aimed to evaluate the performance of cassava tissue culture plantlets using different substrates for rapid multiplication. Transplantation of in vitro nodal segments into three distinct growing substrates: cocopeat (CP), sawdust (SD), and sand (S) were tried in the experiment. These substrates were moistened with either Murashige and Skoog liquid solution or an experimental SAH solution for 60 days. The results showed that survival was significantly influenced by the substrate used, with cocopeat demonstrating the highest rate of more than 90%. The survival rate, shoot length, root numbers, and leaf numbers were collected at the end of one week. The differences in survival rates and growth parameters were significant among substrates ($p<0.001$).

Keywords: Cassava, Cocopeat, Saw Dust, Sand, Semi-Autotrophic Hydroponics (SAH)

Introduction

Cassava (*Manihot esculenta* Crantz) is a vital staple crop, providing daily calories for over a billion people across Africa, Latin America, and Asia (FAOSTAT, 2018) (Wang et al. 2018). Globally, cassava is the fourth most crucial staple food, following rice, wheat, and maize, contributing 2.6% (Rey and Vanderschuren, 2017; Tafesse et al. 2021) to worldwide caloric intake. Utilizing stems as seeds are costly and facilitates disease transmission (Duraisamy et al. 2013). Tissue culture is an effective alternative for mass-producing disease-free plantlets, but high mortality rates upon soil transfer remain a challenge (Short, et al. 1987). These plantlets develop a culture-induced phenotype that cannot withstand the ambient environmental conditions when planted directly in a glasshouse. In vitro cultivation hinders the development of cuticles, epicuticular waxes, and functional stomatal apparatus, leading to excessive stomatal and cuticular transpiration (Durkovic et al. 2009; Fuentes et al. 2007; Joshi et al. 2006). Gradual acclimatization through staged humidity reduction improves survival rates. (Phillips and Garda, 2019; Sutter et al. 1992).

Thorough adaptation prevents high mortality when transferring plants to *ex vitro* conditions (Pospisilova et al. 1999). Semi-autotrophic hydroponics (SAH) is effective for acclimating cassava tissue culture plantlets by adapting them to environmental factors such as temperature, humidity, and light. Unlike traditional tissue culture,

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SAH lacks sucrose, encouraging plantlets to utilize energy sources and develop photosynthetic leaves (Diettrich et al. 1992; Preece and Sutter, 1991). Gradual light exposure enhances epicuticular wax formation, improves stomatal function, reduces transpiration rates, and strengthens water regulation by developing effective stomata (Hasegawa et al. 1973; Rhee et al. 1998; Pospisilova et al. 1999).

A basic approach to disease control is to use uninfected propagules for all new plantings. The mass production of virus-free plantlets from tissue culture, such as pineapples and roses lettuce, employs SAH technology. The substrate and nutrient solution used is the success of stem multiplication in semi-autotrophic hydroponics (SAH). Key nutrients such as calcium, magnesium, iron, and potassium play crucial roles in plant development. Calcium nitrate [$Ca(NO_3)_2$] is a crucial component of the experimental solution, as calcium is vital for cell wall (Hepler et al. 2001), cell membrane (Hocking et al. 2016). structure and integrity, enhancing resistance to pathogens insects (Ranty et al. 2016). In addition to cell walls, Ca_2^+ also stabilizes cell membranes through the interaction with phospholipids (Hepler, 2005). Mg, which is essential for chlorophyll formation, is critical for photosynthetic CO_2 fixation (Cakmak and Kirkby, 2008; Gerendas and Fuhrs, 2013). Iron (Fe) functions as a cofactor for numerous redox reactions, facilitating respiration, photosynthesis, and chlorophyll production (Jeong and Connolly, 2009). Potassium (K), the most prevalent inorganic cation, ensures optimal plant development (Murrell et al. 2021) and assists in cell growth and various other plant functions (Hepler et al., 2001). Potassium (K) plays a vital role in maintaining cellular balance, regulating osmotic pressure, and ensuring equilibrium between cations and anions in the cytoplasm. (Hu et al. 2016). Through these processes, K is involving in control of stomatal movement, cell elongation and distribution of photosynthetic products (Pettigrew, 2008). The present study adopted SAH to improve cost-effectiveness and acclimatization of virus-free cassava plantlets, resulting in higher survival rates and enhanced growth. Using locally sourced substrates and in-house nutrient solutions can further reduce costs.

Materials and Methods

A SAH system was established at ICAR-Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, maintaining an initial temperature of 25-28°C, humidity at 50 g m^{-3}, and a photoperiod of seven hours using a 20 W light. Two popular cassava varieties, H-226 and H-165 were used with 2-5 mm segments excised from 30-35 day-old *in vitro* plants grown on Murashige and Skoog's (MS) basal medium. The SAH method involves placing young nodal cuttings in transparent light boxes with various substrates, moistened with the nutrient solution. Thirty six boxes (22 cm×12 cm×6 cm) containing sterilized substrates, *viz*., coco peat, sawdust and sand, in 12 boxes each were used for the study. The substrates were filled in the boxes to a depth of three centimeters from the bottom (800 ml). Each box lid had 15 holes for aeration. To minimize transpiration and fungal contamination, the lids remained closed for ten days.

The experimental SAH solution components were derived from a pineapple SAH study (Olagunju et al., 2021). The composition was as follows:

Experimental Solution A: 1mM calcium nitrate solution in 1 L distilled water.

Experimental Solution B: Magnesium sulphate (14.7 g), potassium monophosphate (4.08g), potassium nitrate (15.5 g) and ferrous sulphate (0.02 g) dissolved in 1 litre of distilled water.

Table 1. Components of solution B in one litre

Sl. No.	Composition	Quantity (g)
1	Magnesium sulphate	14.7
2	Potassium monophosphate	4.08
3	Potassium nitrate	15.5
4	Ferrous sulphate	0.02

The SAH Solution was prepared by combining 500 ml of solution A and 500 ml of solution B, then diluting to 2 L with water. Solution B was stored in a black container to prevent iron compound oxidation.

The virus-free survival rate of each genotype grown in similar substrate and nutrient solutions was recorded over 60 days. The root and shoot lengths were recorded at 15 and 30 days for each genotype when moistened with MS or experimental solutions. The seedlings were kept in a regulated greenhouse.

Statistical evaluation for each substrate planting was carried out using Data Novia. A three-way ANOVA was employed for statistical comparison, at a significance level of 0.05. Genotype, substrate, and their interactions were treated as fixed effects. When a significant interaction between genotype and substrate was observed, mean comparison was carried out using least significant difference (LSD) test at 0.05 percent level of significance.

Results and Discussion

The present study demonstrated the successful adaptation and rapid multiplication of cassava plantlets. The investigation involved testing various combinations of three different substrates, two nutrient solutions, and two genotypes to determine the optimal conditions for cultivating robust cassava plants using tissue culture techniques. The survival rate of cassava plantlets was mainly influenced by the substrate used, rather than by the interactions between genotype and substrate.

Significant differences ($p<0.05$) in survival rates were observed only among the three substrates. The highest survival rate was recorded for the H-165 genotype in cocopeat, achieving 95% survival when moistened with either the experimental solution or the MS solution. In contrast, for the H-226 genotype planted in cocopeat, the survival rates were 90.4% with the experimental solution and 89.1% with the MS solution, showing no significant difference between them. A similar pattern was observed with sawdust, where there was no significant difference in survival rates for H-226 when treated with either solution. No significant interaction between genotype and substrate was observed in any of the subcultures. This could be due to the nutrient solution comparable to the one that was optimized for root development (Subbarao et al. 2006). The hydroponic system demonstrated high survival rates and consistent growth, even when young stems were used as the source material (Tokunaga et al. 2020). Previous study has shown that nitrogen is an essential nutrient element for plant growth, with a significant impact on plant physiology, yield, and quality (Ding et al. 2022).

Cocopeat had the highest survival rate when moistened with either ES or MS solutions. This result is in agreement with previous findings of Mammy et al. (2024). In contrast, sand showed the lowest survival rate, regardless of whether SAH or MS solutions was used. The poor survival of plantlets in sand could be explained by its physical properties. Several studies have indicated low survival rates for cassava plantlets grown in vermiculite, primarily due to its high water holding capacity, which reduces the amount of air-filled space and hinders root respiration, ultimately affecting the overall plant health (Khan et al. 2021; Shewa et al. 2020). So, vermiculite was not used as a substrate because it lacks the necessary balance between moisture and root aeration, which is crucial for healthy plant growth (Dexter, 2004).

The primary challenge when transferring tissue culture plants to soil conditions is due to the weak vigour of the seedlings. To overcome this, the SAH technique was employed which reduced cassava plantlet mortality, which is often caused by pathogens like *Aspergillus* and *Pythium,* as well as heat shock (Fig. 1).

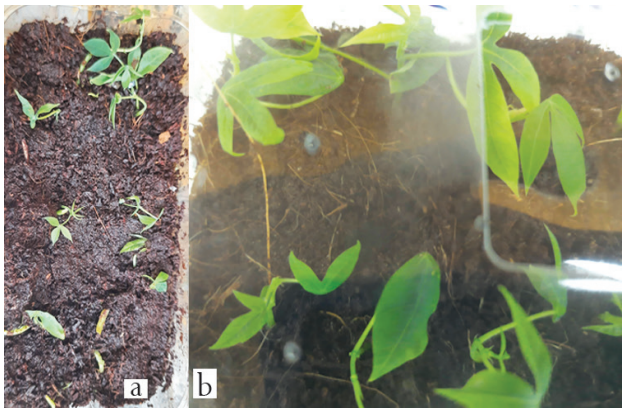

Fig. 1. SAH plantlets (a) After 10 days and (b) After one month

Based on previous research, only the essential elements necessary to develop a nutrient solution for SAH were incorporated in this study. The shock of transplanting tissue culture plants into the soil environment is mainly due to poor seedling vigour (Cuesta et al. 2010). Essential elements were utilized (Olagunju et al. 2021) for the acclimatization of tissue- cultured pineapple. The IITA has developed a nutrient solution to support cassava plantlets in SAH, highlighting the significance of selecting an appropriate nutrient combination for an effective hydroponic system.

Based on ANOVA, there was a significant difference in shoot length among the three substrates. The genotype H-226, when planted on cocopeat exhibited a significantly higher shoot length of 8.5 cm, followed by those planted in sawdust with 7.66 cm when moistened with experimental solution. The genotype H-165 planted on cocopeat also demonstrated a significantly higher shoot length of 7.9 cm, followed by those planted on sawdust (6.6 cm) and sand (1.2 cm), when moistened with the experimental solution. When H-226 was planted onto the substrates and moistened with MS, it showed a higher shoot length in cocopeat (8.1 cm), followed by sawdust (7.2 cm) (Table 1).

Table 1. Response of different cassava varieties to different substrates and nutrient solutions in SAH

Genotype	Substrate	NS	Shoot length (cm)	Root length (cm)	Leaf number	Survival rate (%)
H-165	CP	ES	7.90^{b}	1.90^{b}	3.66^{a}	95.6^{a}
	SD	ES	6.60^{d}	1.30^{cd}	2.33^{b}	68.8^{c}
	S	ES	1.20^{e}	1.33^{e}	0.66^{c}	9.4^{d}
	CP	MS	8.10^{ab}	2.40^{a}	3.66^{a}	95.3^{a}
	SD	MS	6.50^{d}	1.10^{d}	2.66^{ab}	68.7^{c}
	S	MS	1.40^{e}	0.23^{e}	0.33^{c}	9.1^{d}
H-226	CP	ES	8.50^{a}	2.60^{a}	3.33^{ab}	90.4^{b}
	SD	ES	7.66^{bc}	1.66^{bc}	266^{ab}	69.4^{c}
	S	ES	0.30^{f}	0.16^{e}	0.33^{c}	11.5^{d}
	CP	MS	8.10^{ab}	2.40^{a}	3.33^{ab}	89.1^{b}
	SD	MS	7.20^{c}	1.80^{b}	2.66^{ab}	68.4^{d}
	S	MS	1.26^{e}	0.30^{e}	0.66^{c}	10.5^{d}

*Mean values with common alphabets in the superscript in each column does not differ significantly

CP: Cocopeat; S: Sand; SD: Saw dust; MS: Murashige and Skoog's and ES: Experimental solution

A significant difference was observed in the root length of the plants grown in the three different substrates. The highest root length was observed in H-165 planted on the substrate, cocopeat (1.9 cm), followed by that planted on saw dust (1.3 cm) and sand (1.3 cm), and moistened with the experimental solution. Similarly, the highest root length was observed in H-226 planted on cocopeat, followed by that on saw dust when moistened with the experimental solution. The H-165 as well as H-226 plants grown on cocopeat and moistened with MS solution showed higher root length of 2.4 cm. Nitrogen compounds affect the formation and enlargement of cassava storage roots. Given the aerobic nature of cassava, a nitrate-rich nutrient solution was used to stimulate root development. Regarding shoot elongation, H-226 plants exhibited notable differences across the three growing media when treated with ES solution, highest growth in CP followed by S and SD when moistened with the MS solution. A novel hydroponic system utilizing a nutrient solution for cassava, enabling rapid root formation, was reported by Castaneda-Mendez et al. (2017).

Soil-root interactions play a crucial role in plant growth and crop survival (De Dorlodot et al. 2007; Postma and Lynch, 2011). For H-165, the shoot length varied among the three substrates when treated with ES or MS solutions. Like H-226 plants, CP emerged as the optimal substrate, whereas sand provided minimal growth support. This pattern was mirrored in root length, with CP outperforming saw dust and sand. Poor root growth in sand was linked to salinity, reducing crop growth by 15-20% in saline soils (Weimberg et al. 1984). Saline soils, characterized by electrical conductivity exceeding 4 dS m^{-1}, can reduce crop growth by 15-20% (Munns and Tester, 2008). The pH directly affects nutrient availability in the rhizosphere and plant uptake, with macronutrients such as nitrogen, potassium, calcium, and magnesium being most accessible at pH 6.0-6.5. The H-226 plants exhibited greater shoot length than H-165 plants across all substrates and solutions. The poor performance of sand could be attributed to the lack of essential nutrients such as nitrogen (N), phosphorus (P), and potassium (K), which are vital for cassava growth (Byju and Suja, 2020).

The leaf number was higher in H-165 planted in cocopeat and moistened with the experimental solution (3.66) followed by that in saw dust (2.33). After one month, H-165 planted in cocopeat showed 3.66 leaves moistened with MS, whereas it was 3.33 for the H-226 planted under same conditions. Nutrient studies revealed that NO_3^- enhanced early fibrous root vigour (initial 30 d), although roots were sensitive to NH_4^- enriched nutrient solution alone. The introduction of NH_4NO_3 (50-50%) enriched nutrient solution triggered storage root initiation and bulking. Various N sources (NH_4^+, NO_3, and their combination, NH_4NO_3) were examined to enhance plant growth in potatoes. The NO_3^- acclimation system markedly improved transgenic line survival rates, with rapid root development boosting the acclimation efficiency (Castaneda-Mnedez et al. 2017).

The interaction between genotype and substrate was significant, indicating that the choice of substrate influenced each genotype differently. Most plants primarily use nitrate (NO_3^-) as their N source (Wang et al. 2003), which regulates plant growth and development (Fenchel et al. 2012). Cassava absorbs N as NO_3-, which is then assimilated into organic N in the shoot through enzymatic action. Research has shown that introducing NO_3^- to N-starved seedlings strongly induces all the genes directly required for NO_3^- assimilation, including the N metabolism enzyme gene (Gowri et al. 1992; Scheible et al. 2004; Wang et al. 2003). Root strength was strongly correlated with cellulose content, indicating that plants grown under high flow rates developed more robust and compact roots.

Conclusion

The Semi-Automatic Hydroponic (SAH) technology offers an advantage compared to traditional propagation methods by enabling the rapid production of large quantities of planting materials and better acclimatization of tissue culture plants. Overall, the results indicate that the choice of substrate significantly impacted the performance of cassava plantlets in the SAH system, while the genotype had a lesser impact. Cocopeat demonstrated the most effective growing medium, supporting faster growth, increased plant height, greater leaf numbers, and higher survival rate compared to sawdust and sand. On the other hand, substrates such as sawdust and sand exhibited poorer performance in terms of growth parameters and multiplication rates.

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