AN EFFICIENT *IN VITRO* PROPAGATION PROTOCOL FOR A SUSTAINABLE SUPPLY OF *CASSAVA*

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Millions of people in many countries of the world depend on Cassava (*Manihot esculenta*) for food security, as it thrives in marginal soils and can help combat hunger. However, slow reproduction rates hinder the adoption of improved varieties. A protocol for *in vitro* propagation of Cassava using stem nodes was developed. Sterile stem nodes were cultured on Murashige and Skoog (MS) medium with different concentrations of naphthalene acetic acid (NAA) and benzyl adenine (BA). The optimal growth medium was MS medium supplemented with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA, producing two shoots per explant. The axillary buds generated during the establishment stage were cultivated on MS medium supplemented with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA in order to multiply them. The best multiplication results were obtained from the fourth subculture. Indole-butyric acid (IBA) at 1.0 mg l^{-1} and 0.5 mg l^{-1} indole-acetic acid (IAA) were added to MS medium for rooting. The seedlings were acclimatized successfully in a 1:1:1 mixture of sand, peat and silt, achieving 100% survival rate and showed no phenotypic differences during their growth period under greenhouse conditions.

Keywords: *Manihot esculenta, micro*propagation, acclimatization

INTRODUCTION

Cassava (*Manihot esculenta)* is a perennial vegetatively proliferating bush, adaptable to a variety of soil types, belonging to the Euphorbiaceae family, grows under temperature and precipitation conditions that fluctuate between 10°C and 40°C and between 900 mm and 2000 mm, respectively. Cassava is grown in sub-tropical countries between 30° N and 30° S and up to 1,800 meters above sea level, including an area of more than 13 × 106 hectares. The trim is mostly made for its tuberized roots, which contain 80% starch as dry matter (El-Sharkawy, 2004). After rice, wheat, and maize, Cassava is the fourth most important staple in the world and is essential to the diets of more than a billion people (FAO, 2000). Furthermore, the main obstacle to the new varieties' broad marketing and higher Cassava production

is the lack of high-quality, true-to-type planting supplies (Escobar et al., 2006). In Africa, where climate change is lowering cereal crop yields, Cassava is the best candidate crop for attaining food security since it can grow in a range of agro-ecologies, including moderately fertile soils, and it is drought tolerant (Feyisa, 2021). A large portion of their population tends to be unaware of the importance of Cassava. These days, in addition to being used for human and animal use, Cassava and its derivatives are now used to address mechanical needs such the manufacturing of paper, sticks, biofuel, and materials (Saelim et al., 2009). The lack of essential traits in the germplasm has hindered the advancement of the Cassava germplasm through traditional breeding methods; therefore, more recent methods of *in vitro* engendering and hereditary change advertising have more noteworthy trust for Cassava advancement (Munyikwa et al., 1998). One of the key elements of plant biotechnology nowadays is plant tissue culture. Additional examples of its strategies include the creation of auxiliary metabolites, *ex-situ* preservation of profitable germplasms, recovery and duplication of hereditarily controlled predominant clones, and rapid clonal proliferation (Gupta and Ibaraki, 2006). Using plant tissue culture, *in vitro* proliferation is a common technique for vegetative development that has a number of benefits over conventional plant engendering methods. One is that an exceptionally high number of seedlings can be produced on a little area (Hopkins, 2007).

In order to rapidly generate a large number of virus-free plantlets, the aim of this work was to develop a basic technique for the *in vitro* cultivation of virus-free Cassava plants. By 2050, farm output of crops like Cassava would need to be more than triple. Therefore, in order to increase supply chain efficiency and decrease waste, the globe needs to find innovative ways to boost production. Innovation and productivity-boosting technology are necessary to address such issues and boost agricultural productivity (Ray et al., 2013).

MATERIALS AND METHODS

This investigation was carried out in Tissue Culture Unit, Department of Genetic Resources, Desert Research Center, Cairo, Egypt as an activity of the research program "Sustainable development of non-traditional Cassava crop as an alternative to strategic crops (wheat) using modern technology under New Valley conditions" funded by Desert Research Center. A promising protocol was established for the *in vitro* propagation of Cassava virus free plants.

1. Plant Materials

The stem nodes were used as plant materials. The plant parts were collected from Cassava imported from Thailand, one of the most important countries famous for Cassava cultivation, and were cultivated at the New

Valley Sustainable Resources Development Center, Desert Research Center, Kharga, New Valley Governorate, Egypt.

2. Explant Preparation

To prevent tissue browning, the explants were washed five times with a sterile antioxidant solution containing 150 mg l^{-1} citric acid and 100 mg l^{-1} ascorbic acid. Following a 24-hour slow-flowing washing with tap water, the explant materials were surface-sterilized with 15% Clorox (5% sodium hypochlorite) containing three drops of Tween-20 for 20 minutes. After that, sterile distilled water was used to rinse them three times. Explants that had been sterilized served as test subjects.

3. Culture Medium and Conditions

Throughout this study, Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) (Duchefa, Harrlem, the Netherlands) was used. It was supplemented with vitamins and 3% (w/v) sucrose. It was then adjusted to pH 5.8 ±1 and solidified with 0.27% (w/v) phytagel (Duchefa, Harrlem, the Netherlands). Finally, it was autoclaved for 15 minutes at 121°C. Growth regulators were used at different concentrations (mg l^{-1}) according to the growth stage. Among these were naphthalene acetic acid (NAA), 6-benzylamino purine (BA), indole acetic acid (IAA) and indol-3-butyric acid (IBA) (Sigma Cell Culture, min. 90%, St. Louis, USA). Every culture was kept at 25±2°C under cool white fluorescent tubes (Toshiba F 140 t ad/38). Data were gathered following a four-week culture period. Additionally, four weeks after each incident, subcultures for the multiplication stage were conducted.

4. *In Vitro* Propagation

This protocol includes four sequential stages, i.e., establishment, multiplication, rooting and acclimatization stages.

4.1. Culture Establishment

Different concentrations of BA (0.5, 1.0 and 2.0 mg l^{-1}) and 0.1 mg l^{-1} NAA were added to MS culture medium to perform the establishment of the explants and initiate the shoots. Then, the survival percentage, growth induction percentage, average number and length (cm) of axillary shoots per explant were recorded after four weeks.

4.2. Shoots Multiplication

Fresh MS medium supplemented with different concentrations of BA at 1.0, 2.0, 3.0, and 4.0 mg l^{-1} in combination with different concentrations of NAA (0.1, 0.2, and 0.3 mg l^{-1}) was used to cultivate uniform explants obtained from the establishment stage that were roughly 2-3 cm long. After four weeks, the number of newly generated axillary shoots and their mean length (cm) per explant were recorded for five successive subcultures.

4.3. Root Formation

To achieve root formation, obtained shoots from the multiplication stage (about 2-4 cm in length) were removed and recultured in MS medium supplemented with different concentrations of IBA (0.5, 1.0, and 1.5 mg l^{-1}) and IAA at 0.5, and 1.0 mg l^{-1}. After four weeks, the well-rooted plantlets were transferred to MS medium free of growth regulators as a control medium. The percentage of root induction, the number of roots/explant, and the average length of roots (cm)/explant were measured after 10 weeks of culture.

4.4. Acclimatization

To increase the effectiveness of the roots, the rooted shoots (neoformed plantlets) were left in their media for 14, 24, and 34 days prior to acclimatization without subculturing. To get rid of any medium residue, the neoformed plantlets were thoroughly cleaned. After being treated with 1 g l^{-1} of Benelet (an antifungal), the neoformed plantlets were placed in pots with clear plastic bags over them in a 1:1:1 sand, peatmoss, and silt (v/v) combination. The plantlets were transferred to a greenhouse with a temperature of 27±1°C and a relative humidity of 80–90% in order to maintain high humidity. The clear plastic bags were taken out after 20 days to make room for more growth.

5. Statistical Analysis

Five replicates were used for each treatment in each experiment, which was carried out using a completely randomized design using factorial experiment architecture (Gomez and Gomez, 1984). The ANOVA statistical analysis tool was used to analyze the variance of the data. Significance was established at 0.05 (Duncan, 1955) using the least significant difference (L.S.D.). In this regard, probability levels expressed in percentages were used usually.

RESULTS AND DISCUSSION

1. Culture Establishment

This stage is considered a very important stage because the success of this stage affects the success and effectiveness of any tissue culture protocol for any plant, as this stage depends on the interaction between the sterilization method used and the growth regulators under study (Pasternak and Steinmacher, 2024). At this stage, the part to be grown must be sterilized using tissue culture superficially to get rid of any traces of microbial contaminants and grown in nutrient media. Many different sterilization protocols have been used in sterilizing Cassava, but the most commonly used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol, and mercuric chloride (Sessou et al., 2020).

Many research works have shown that MS medium is the most common medium for Cassava micropropagation. Different growth regulators

and different concentrations of cytokinins were used, alone and in combination with auxins to induce Cassava shoot to start growing (Onwubiku and Onuoch, 2007 and Beyene, 2009). There are also reports on the important role of cytokinin in Cassava micropropagation (Sukmadjaja and Widhiastuti, 2011 and Demeke et al., 2014).

Different concentrations of BA (0, 1 and 2 mg l^{-1}) in combination with NAA (0, 0.1 and 0.2 mg l^{-1}) were used. Then, the percentage of survived explants, growth induction percentage, and number of axillary shoots on each explant and average lengths of the resulting axillary shoots were measured. the highest percentage of survived explant was recorded on MS medium supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA (100%). Also, the same medium composition gave the highest mean number of axillary shoots/explant and mean length of axillary shoots of 2.0 and 7.5 cm, respectively (Table 1 and Fig. 1). While MS medium free of growth regulators recorded the lowest values of all parameters. Abd-Alla et al. (2013) used the same growth regulators at the same stage of the micropropagation of Cassava.

2. Shoots Multiplication

In this study, MS medium supplemented with 0.1 mg l^{-1} NAA in combination with 1.0 mg l^{-1} BA was the most effective medium for the multiplication of Cassava for five success transfers (Table 2). The subculture was done for a fixed period of four weeks. Mean number and length (cm) of axillary shoots/explant were taken after each subculture. The results in Table (2) show that the mean number of axillary shoots/explant increased directly with the number of transfers, as in the first subculture, it was 2 and in the fifth subculture it was 4.6 per explant (Fig. 2). These results were completely opposite when taking the readings for the mean length of axillary shoots (cm), as it decreased with the increase in the number of transfers, as it was 8.07 cm in the first subculture and 4.02 cm in the fifth subculture.

This phenomenon can be attributed to the physiological responses of the explants to the growth regulators and the medium conditions provided during the culture periods. As the number of transfers increased, the explants may have experienced a heightened production of axillary shoots, indicating a positive response to the tested medium composition (MS medium supplemented with 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA). The increase in the mean number of axillary shoots/explant from 2 to 4.6 suggests that the plants were able to proliferate effectively under the specified conditions.

Conversely, the reduction in the mean length of axillary shoots from 8.07 cm to 4.02 cm over the same period raises important considerations regarding the balance between shoot proliferation and elongation. This decline in shoot length may indicate a shift in resource allocation within the plant tissues, where energy and nutrients are preferentially directed towards the formation of new shoots rather than the elongation of existing ones. It is also possible that repeated transfers and subculturing could have led to a form of

stress or adaptation in the explants, resulting in shorter but more numerous shoots.=

Table 1. *In vitro* establishment of Cassava *(Manihot esculenta)* stem nodes cultured on MS medium supplemented with different combinations of BA and NAA.

Parameters	NAA conc. (mg l^{-1})	BA conc. (mg l^{-1}) 0	1	2
Survived explants (%)	**0.0**	73 c	80 bc	80 bc
	0.1	73 c	100 a	93 ab
	0.2	73 c	93 ab	93 ab
Growth induction (%)	**0.0**	73 b	87 ab	87 ab
	0.1	83 ab	100 a	93 ab
	0.2	80 ab	93 ab	93 ab
Mean number of axillary shoots/explant	**0.0**	1.4 a	1.5 a	1.5 a
	0.1	1.6 a	2.0 a	1.9 a
	0.2	1.6 a	1.9 a	1.9 a
Mean length of axillary shoot (cm)	**0.0**	6.03 b	6.92 ab	6.9 ab
	0.1	6.50 ab	7.50 a	7.1 ab
	0.2	6.50 ab	7.10 ab	7.1 ab

Data were recorded every four weeks of culture with a total of 5 replicates of 10 explants per treatment. Means having the same letter within a column are not significantly different at 0.05 level of probability.

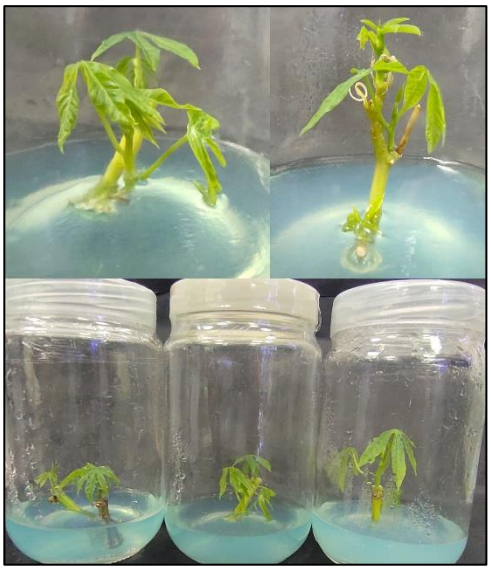

Fig. (1). Established stem nodes of Cassava *(Manihot esculenta)* on MS medium supplemented with 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA.

Table 2. Effect of MS medium supplemented with 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA on multiplication of Cassava *(Manihot esculenta)* through five successive subcultures.

Parameters	No of subculture				
	1st	2nd	3rd	4th	5th
Mean number of axillary shoots/ explant	2.00 d	3.20 c	3.80 b	4.40 a	4.60 a
Mean length of axillary shoots (cm)	8.07 a	5.74 b	4.44 bc	4.15 c	4.02 c

Data were recorded every four weeks of culture with a total of 5 replicates of 10 explants per treatment. Means having the same letter within a column are not significantly different at 0.05 level of probability.

Fig. (2). Multiplication of Cassava *(Manihot esculenta)* on MS medium supplemented with 0.1 mg l^{-1} NAA +1 mg l^{-1} BA.

3. *In Vitro* Rooting Stage

Rooting of *in vitro* developed shoots of Cassava consists of rooting plants under ideal conditions that are completely free of germs and contamination, as these conditions contribute greatly to the success of the plants in forming a strong and stable vegetative system. The success of the rooting process depends mainly on the medium being free of contaminant, and this requires the use of advanced techniques to ensure the best results. Despite the high prices of the growth regulators used in this process, which makes the rooting process relatively expensive, the importance of rooting *in vitro* remains very prominent. It allows the production of plants with a strong and effective root system, which makes them well qualified to enter the

acclimatization process in the greenhouse afterwards. Therefore, it can be said that investing in this process is essential to achieve sustainable success in Cassava cultivation.

The study comprehensively investigated the effects of different concentrations of the auxins; IBA and IAA when incorporated into MS medium. It specifically examined how these variations in concentration influenced key parameters, including the percentage of plants that were successfully rooted, the total number of roots produced, and the length of these roots measured in centimeters (Table 3). This assessment was conducted after four weeks of cultivation.

Table 3. Effect of different concentrations of IBA, IAA and their combinations on Cassava *(Manihot esculenta) in vitro* rooting.

Parameters	IAA conc. (mg l^{-1})	IBA conc. (mg l^{-1}) 0	0.5	1	1.5
Roots induction (%)	**0.0**	0 c	80 ab	90 a	60 b
	0.5	80 ab	90 a	100 a	60 b
	1.0	60 b	60 b	90 a	60 b
Mean number of axillary roots / explant	**0.0**	0.0 c	2.5 ab	2.6 ab	1.6 ab
	0.5	2.6 ab	2.5 ab	2.7 a	2.1 ab
	1.0	2.2 ab	1.4 b	2.4 ab	2.3 ab
Mean length of axillary roots (cm)/ explant	**0.0**	0.00 d	6.09 ab	6.23 a	4.00 abc
	0.5	6.16 ab	6.22 a	6.32 a	4.05 abc
	1.0	5.23 abc	4.50 abc	3.27 bc	2.85 cd

Data were recorded after 10 weeks of culture with a total of 5 replicates of 10 explants per treatment. Means having the same letter within a column are not significantly different at 0.05 level of probability.

Table (3) indicates that there were variations among the treatments. After 10 weeks of culture, the highest percentage of root induction (100%) was achieved with 0.5 mg l^{-1} IAA and 1.0 mg l^{-1} IBA, whereas no rooting was achieved using MS medium free from growth regulators. The same result was reached for the number of roots, where 2.7 roots were produced on the same concentration of IBA and IAA (0.5 mg l^{-1} IAA and 1.0 mg l^{-1} IBA). Also, when studying the length of root, the extreme root length (6.3 cm) was recorded on the same medium composition, followed by a 6.23 cm with insignificant difference (on MS medium supplemented with 1.0 mg l^{-1} IBA and MS medium supplemented with 0.5 mg l^{-1} of both IAA and IBA. So, it could be concluded that MS medium supplemented with 0.5 mg l^{-1} IAA and 1.0 mg l^{-1} IBA, achieved the best values 100, 2.7 and 6.3 for percentage of rooting, roots number and length of roots, respectively (Fig. 3). On the other hand, free growth regulators medium gave no response at all.

Fig. (3). *In vitro* rooted plantlets of Cassava *(Manihot esculenta)* after 10 weeks of culture on MS medium supplemented with 0.5 mg l^{-1} IAA and 1.0 mg l^{-1} IBA.

Thus, it can be concluded that IBA and IAA were more effective in initiating root growth than the medium containing IBA alone. These results are nearly in line with those of Kalidas and Mohan (2009) on *Phyllanthus urinaria*, Shrivastava and Banerjee (2008), Singh et al. (2010) and Khemkladngoen et al., (2011) on Jatropha. One possible explanation for this could be that IBA promotes the conjugation of endogenous IBA with amino acids, which results in the production of certain proteins needed for root initiation (Xavier et al., 2012). However, some research has shown that IBA, IAA, and NAA combinations were more effective at starting roots than the effects of each of them alone such as Catapan et al. (2000) on Carolina rose leaves, Kumar et al. (2011) and Sharma et al. (2011) on Jatropha. While the cellular levels of auxin, in turn contribute to the regulation of gene expression that defines cell fate and pharmacological or genetic disruptions of auxin movement dramatically impacts root patterning (Aida et al., 2004 and Overvoorde et al., 2010). Similar effects of IBA were also observed in many plans (Sakila et al., 2007) such as *Capsicum annum* (Agarwal et al., 1989) and *Prunus* sp. (Mante et al., 1989).

4. Acclimatization Stage

Acclimatizing plants to free-living conditions after *in vitro* cultivation is a crucial step in tissue culture. A successful tissue culture protocol is evidenced by a high frequency of tissue culture-derived seedlings thriving in soil. Transferring Cassava seedlings with established roots to a greenhouse is

vital for their growth and survival. When Cassava plantlets placed in an optimal soil mix of peat moss, sand, and silt at a 1:1:1 ratio, seedlings could achieve high survival rates, potentially reaching 100% (Fig. 4). Successful acclimatization relies on factors such as the soil mixture and the duration of exposure, along with careful control of greenhouse conditions. These findings align with Abd-Alla et al. (2013) in their study on Cassava micropropagation and the acclimatization success rate mirrors that reported by Sessou et al. (2020) with different soil mixtures.

Fig. (4). Acclimatized plantlets of *Cassava (Manihot esculenta)* after 12 weeks from transfer to the greenhouse.

CONCLUSION

No significant differences were observed among treatment groups of all stages of the *in vitro* propagation of Cassava, and plantlets exhibited normal growth patterns in all conditions. This suggests a connection between internal physiological mechanisms and root health, with robust root systems crucial for acclimatization. A major challenge in Cassava production is the slow multiplication rate, but tissue culture techniques offer a solution. Key factors influencing these methods include genetic variation, explant type, phytohormone concentration, and regeneration strategies. Nodal segment explants are effective for producing true-to-type plantlets. High cytokinin levels combined with low auxin concentrations enhanced shoot proliferation, while IAA and IBA are the most effective auxins for *in vitro* rooting of Cassava. Due to the importance of Cassava in providing alternative food sources for patients with wheat gluten sensitivity, it is necessary to consider future plans that allow for the commercial implementation of this protocol. This implementation will have a positive impact on the production of Cassava seedlings, which will lead to meeting the increasing nutritional needs of this group of people. In addition, this approach will contribute to bridging the food gap that some communities suffer from, and will help reduce dependence on

imports, thus preserving the hard currency that is spent on purchasing these alternative foods. It is important to promote these initiatives to achieve food security and improve the quality of life for patients who need appropriate nutritional options.

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بروتوكول فعال لإكثار الكاسافا معمليًا لتوفير مصدر مستدام من النبات

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يعتمد ملايين الأشخاص في العديد من دول العالم على الكاسافا لتحقيق الأمن الغذائي، حيث تزدهر في التربة الهامشية ويمكن أن تساعد في مكافحة الجوع. ومع ذلك، فإن معدلات اكثار النبات البطيئة تعيق تبني الأصناف المحسنة. لذا تم تطوير بروتوكول لإكثار الكاسافا في المختبر باستخدام العقد الساقية، مع أفضل معدل بقاء وأقل تلوث باستخدام ٢٠٪ كلوروكس لمدة ١٥ دقيقة. تمت تربية العقد الساقية المعقمة على بيئة موراشيج وسكوج (MS) مع تركيزات مختلفة من البنزيل أدينين مع نفثالين حمض الخليك. وكان وسط النمو الأمثل على بيئة موراشيج وسكوج مضافًا إليها ١,٠ مجم لتر من البنزايل أدينين و ٠,١ مجم لتر من نفثالين حمض الخليك، مما أسفر عن متوسط ٢ برعم لكل نبات. ولتضاعف البراعم الإبطية الناتجة من مرحلة التأسيس تم زراعتها على بيئة موراشيج وسكوج تحتوي على ١,٠ مجم لكل لتر من البنزيل أدينين مع ٠,١ مجم لكل لتر حمض نفثالين حمض الخليك من خلال خمس زراعات فرعية متتالية وأعطت الزراعة الفرعية الرابعة أفضل النتائج للإكثار. وللتجذير تم استخدام بيئة موراشيج وسكوج مضافًا إليها ١,٠ مجم لكل لتر اندول حمض البيوتيريك و٠,٥ مجم لكل لتر اندول حمض الخليك. وتأقلمت الشتلات بنجاح في خليط ١:١:١ من الرمل والجفت والطمي، محققة بذلك معدل بقاء للنباتات بلغت ١٠٠٪ ولم تظهر أي اختلافات مظهرية أثناء فترة نموها تحت ظروف الصوبة.