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A simple hydroponic system and the effect of nitrogen source on the acclimation of in vitro Cassava (Manihot esculenta)

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Abstract:	Plant tissue culture technology is being widely used for large-scale, rapid, clonal multiplication and genetic transformation in cassava. The main limitation of these technologies is the period of acclimation of the fragile in vitro plants after they have been multiplied or regenerated. Most losses of in vitro plantlets occur when the plantlets are moved directly from the test tubes to the natural soil. Our aim was to design simple, rapid, low-maintenance hydroponic system to improve the rapid acclimation process of in vitro plants. In this paper, we have developed a simple hydroponic system to accelerate rapid cassava acclimation and multiplication. This system considerably increased the survival percentage of in vitro and/or transgenic lines and reduces the time requirement for multiplication by hydroponic acclimation. In order to assess the effectiveness of the acclimation of seedlings on their establishment, we analyzed plant growth and field survival rate with response to different N forms using different cassava accessions. The relationships between the type of N forms and plant survival were also analyzed. NO3- and NH4NO3 increased plant growth and root vigor compared to sole NH4+ and water treatments. The greenhouse and field survivability of N hardened plants, including transgenic lines, were significantly different in growth and development. We present a simple NO3- hydroponic acclimation system that can be quickly and cheaply constructed and used by the cassava community around the world. The efficiency of our proposed N hydronic acclimation system is validated in the transgenic development pipeline which will enhance the cassava molecular breeding.

A simple hydroponic system and the effect of nitrogen source on the acclimation of *in vitro* Cassava (*Manihot esculenta*)

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Abstract

Plant tissue culture technology is being widely used for large-scale, rapid, clonal multiplication and genetic transformation in cassava. The main limitation of these technologies is the period of acclimation of the fragile *in vitro* plants after they have been multiplied or regenerated. Most losses of *in vitro* plantlets occur when the plantlets are moved directly from the test tubes to the natural soil. Our aim was to design simple, rapid, low-maintenance hydroponic system to improve the rapid acclimation process of *in vitro* plants. In this paper, we have developed a simple hydroponic system to accelerate rapid cassava acclimation and multiplication. This system considerably increased the survival percentage of *in vitro* and/or transgenic lines and reduces the time requirement for multiplication by hydroponic acclimation. In order to assess the effectiveness of the acclimation of seedlings on their establishment, we analyzed plant growth and ~~field~~ survival rate with response to different N forms using different cassava accessions. ~~The relationships between the type of N forms and plant survival were also analyzed~~. NO_3^- and NH_4NO_3 increased plant growth and root ~~vigor~~ compared to sole NH_4^+ ~~and~~ water treatments. The greenhouse and field survivability of N hardened plants, including transgenic lines, were significantly different in growth and development. We present a simple NO_3^- hydroponic acclimation system that can be quickly and cheaply constructed and used by the cassava community around the world. The efficiency of our proposed N hydronic acclimation system is validated in the transgenic development pipeline which will enhance the cassava molecular breeding.

Abbreviation

Hydroponic Solution (HS)

International Center for Tropical Agriculture (CIAT)

Nitrogen (N)

RATI (Recipient for Automated Temporary Immersion)

Days After Transplanting (DAT)

Fresh weight (FW)

Maximum root length (MRL)

Maximum stem length (MSL)

Root thickness (RT)

Stem thickness (ST)

Alive leaves (AL)

Dead leaves (DL)

Keywords

Nitrogen, Hydroponic system, Acclimation, Cassava (*Manihot esculenta*), *in vitro*

Introduction

Cassava (*Manihot esculenta*), a tropical root crop originated from Amazonia, is a staple food source for an estimated 700 million people (Wang et al. 2014; http://faostat.fao.org), making it an important storage root crop worldwide. More than one tenth of the world's population relies on this food source. In tropical countries, the main caloric intake is only from maize and rice. (Roca et al. 1992, Saunders 2013). The most important constraints limiting the expansion of cassava production globally is due to lack of planting the material with appropriate quality, quantity at the right time. The circulation of infected planting material remains a major cause of the spread of pests and diseases in cassava.

The tissue culture technology has the potential to solve this issue. Tissue culture has been effectively used to eliminate viruses and other systemic diseases from elite cassava vegetative materials (Roca and Mroginski 1991; Jorge et al. 2000). This has allowed exchange and conservation of rejuvenated propagation materials, which have higher yields than the same varieties propagated for successive years in the field (Kassianof 1992). However, one of the major limitations for a wider adoption of this technique in developing countries is the unavailability of a procedure for acclimation and multiplication of the tissue culture plantlets before final transplanting at the production sites. Although reports are available on *in vitro* acclimation of cassava in the developed world, the protocols are tricky and expensive to implement in developing countries since the technology is capital, labor and energy-intensive (Ahloowalia et al. 2004).

Cassava is also widely used for genetic modification and it is believed that transgenic technologies offer the key to unlocking the full potential of the crop (Liu et al. 2011). Recent

progress in cassava transformation has allowed the robust production of transgenic cassava even under suboptimal plant tissue culture conditions (Liu et al. 2011; Zainuddin et al. 2012). However, a major issue is slow growth rate under controlled environmental conditions. Plants derived from transformation are very delicate and substantially require greater care and handling than conventional *in vitro* plants derived from tissue culture and the acclimation process is frequently associated with high percentage of losses from the death of valuable transgenic lines. So far, RATI (Recipient for Automated Temporary Immersion) systems have been widely used to multiply cassava materials *in vitro*, but this process is inevitably slow and associated with heavy losses (Ospina et al. 2007).

Most of the losses of plantlets occur during transplanting; that is, when plantlets are moved from a test tube to a plastic bag, filled with soil and further transferred to a real field. This direct transplanting is very sensitive for cassava. If the transfer is not carried out with special care, the percentage loss will be very high (from 50 to 95 %) (Ospina et al. 2007). It was also reported that transplanting shock in the soil environment is mainly due to the poor seedling vigor (Cuesta et al. 2010) and root growth (Ospina et al. 2007), especially when the plants are coming from *in vitro* origin, the proper nutrient acclimation system may help to improve seedling performance under harsh environment.

Nitrogen (N) is a major nutrient for plant growth and is taken up by the roots. There are two types of sources: nitrate (NO_3^-) or ammonium (NH_4^+). Several studies have evaluated the effect of nutrient acclimation on field performance of tree seedlings (Vilagrosa et al. 2003; Trubat et al. 2008; Cuesta et al. 2010). Seedling performance depends on ~~seedling~~ morphological and physiological traits, which can be determined to a greater extent by cultivation practices in the nursery (Villar-Salvador et al. 2004). Several studies have reported a positive relationship between transplanting performance of tree seedlings and plant N concentration (Villar-Salvador et al. 2004; Oliet et al. 2009). This indicates that the nutrient composition for seedlings can play an important role in transplanting performance (Salifu and Timmer 2003). The aforementioned associations offer some insights into potential physiological mechanisms involved in the superior transplanting performance of larger-sized or high N concentration seedlings as compared to smaller-sized or lower N seedlings. Several authors have reported the influence of N forms on plant growth and root vigor (Zhang et al. 2007; Ogawa et al. 2014). NH_4^+ supplied as the sole N source inhibited plant growth compared to ~~a mixture of~~ NH_4NO_3 and sole NO_3^- (Feil 1994;

Walch-Liu et al. 2000, Britto and Kronzucker 2002). Britto and Kronzucker (2002) reported NH_4^+ to be toxic compared to NO_3^- for growth of many plants including some Euphorbiaceae species. Plant growth seems to improve when a combination of NH_4^+ and NO_3^- is taken up by the plant. Different N sources (NH_4^+, NO_3^-) and their combination (NH_4NO_3) were tested to improve the plant's growth in rice (Qian et al. 2004; Zhang et al. 2007; Ogawa et al. 2014), in tomato (Barker 1999; You and Barker 2002) and also in potato (Goins et al. 2004; Gao et al. 2014). However, which N source better fits to cassava root growth and development is still unclear. To our knowledge, N acclimation for *in vitro* cassava using hydroponic systems has not been evaluated elsewhere. Our objectives are: (1) to clarify how different N sources used in hydroponics affect the cassava seedling vigor, (2) to examine if the variation responses of cassava plantlets in hydroponic solutions are genotype dependent, and (3) to analyze the relationship between the morpho-physiological changes, resulted from N acclimation, and the seedling's field survival. Developed system to improve the survivability will be helpful to accelerate the multiplication and propagation for cassava community all over the world including developing countries.

Plant materials

Five cassava cultivars viz., TMS60444, SM1219-9, SM3770-12, GM5205-36 and GM4512-5 were used in this study to establish the N acclimation system under greenhouse level. These materials are from the International Center for Tropical Agriculture (CIAT) collection. TMS60444 is widely used for cassava transformation (González et al. 1998; Ubalua 2015). SM1219-9 is used as Cassava frogskin disease tolerance variety (Alvarez et al. 2009) and SM3770-12, GM5205-36 and GM4512-5 were known as high beta carotene varieties (personal communication from CIAT cassava collection). To validate this system, we have also used several genetically transformed cassava lines (TMS60444) carrying early flowering and herbicide resistance genes from the CIAT Genetic Transformation Platform.

Description of the floating hydroponic system at greenhouse

We have designed a very simple, low cost, conventional floating hydroponic system, consisting of a plastic growing tank (55 cm x 36 cm x 23 cm; INDUSTRIAS ESTRA SA, Colombia) and Croydon sponge pieces with pore density of 26 ppi (2 cm x 2 cm x 2 cm; Almacén Washington,

Cali, Colombia) to hold *in vitro* cassava plants (Fig. 2-I). The *in vitro* cassava plantlets from the test tube were placed at the center in a cut made on the topside of the sponge block and was floated in the hydroponic solution. Plastic growing tanks and sponges to hold *in vitro* plants used here are readily available in local markets. Food container tanks are also available in food package stores while the sponge is also available in local stores providing greenhouse supplies.

Plant materials preparation and N acclimation **solutions**

The floating hydroponic system was established in a greenhouse with an average air temperature of 30 °C, an average relative humidity of 45 % and natural light conditions. Experiments in this study were conducted three times. The general workflow for the cassava hydroponics system is summarized in Fig. 1 and 2. *In vitro* cassava propagation was conducted under tissue culture growth room facilities at the CIAT transformation platform. In order to obtain cassava plantlets for standardization, shoot tips were excised (about 2 cm long) from the mother plants which were maintained in the tissue culture lab. Then sterile, excised shoot tips were cultured individually in mayonnaise glass jars (5 cm x 11.5 cm) containing rooting medium (17N) supplemented with 0.028 mg/L of GA3 (gibberellic acid), 0.053 of ANA (1-Naphthaleneacetic acid) and 2.0g/l Gelrite (Fig. 1c), and also containing instead 1/3 of MS salts plus 25 mg/l Plantex® (fertilizer N/P/K 10:52:10). The other components were kept constant (Chavarriaga-Aguirre et al. 2016). The shoot tips were incubated for about six weeks under controlled conditions with a photoperiod of 12 hours light and 12 hours darkness and a temperature of 28 ± 2 ºC (Fig. 1d), and then 6 - 7 cm long uniform *in vitro* plantlets were used for the hydroponic acclimation experiment (Fig. 1e).

The basal nutrient solution used for acclimation was the same as described in Ogawa et al. (2014) (Supplemental Table S1). In order to test the effect of N forms on acclimation, basal nutrient solution, enriched with different forms of N (NH_4^+ as $(NH_4)_2SO_4$, NO_3^- as KNO_3 and NH_4 NO_3), with concentration of 500 µM, were used as sufficient N level (Obara et al. 2011). In addition to N treatments we were also used water treatment as a control. The pH of the hydroponic solution (HS) was monitored daily and the HS was replaced every week.

N acclimation **work flow and trait measurement in greenhouse**

Six weeks old *in vitro* cassava plantlets were transferred from the tissue culture laboratory to a greenhouse for further N acclimation. Initially plantlets were allowed to adapt for one week under greenhouse conditions (Fig. 2-I). After one week of adaptation, plantlets were allowed to float in the N acclimation solutions using a sponge (Fig. 2-I). During the first three days of floating, the water tanks that held the plantlets were covered with a wet white cloth to avoid direct sunlight (Fig. 2-I).

To verify that enough acclimation and acclimation occurred, *in vitro* plantlets were kept around five weeks under the N acclimation HS (NH_4^+, NO_3^- and 50:50 mixture of both NH_4^+ and NO_3^- HS). Every week during HS replacement, we also measured fresh weight (FW), maximum root length (MRL), maximum stem length (MSL), root thickness (RT), stem thickness (ST) and number of alive and dead leaves (AL And DL respectively) of each plantlet. At five weeks, in addition to a routine measurement, we also measured chlorophyll content using a SPAD-502 chlorophyll meter (Konica Minolta Inc., Tokyo, Japan). At the end of the experiment, the survival rate in greenhouse conditions was calculated as the final number of alive individuals in each HS treatment divided by the total number of plantlets introduced per genotype. Insecticide (Lorsban® 10D) and fungicide (Banrot® 40WP) were applied to control pests and fungi to avoid contamination throughout the course of the experiment.

Survival test at field conditions

The survival test of hardened cassava plants was conducted under CIAT field conditions in Cali, Colombia. The texture of the CIAT soil was silt loam (Sand; 18.9%, Clay; 65.4% and Silt; 15.7 %). After five weeks of growth in HS, whole plants of each genotype were transplanted directly to the field at a spacing of 100 x 150 cm. 15 days after transplanting (DAT), survival rate was recorded as the number of plants survived divided by number of transplanted plants.

Method validation at lab level using the best N source

In order to test the efficiency of this system, we introduced N acclimation at laboratory level using NO_3^- as ~~a~~ N source based on the results obtained from previous greenhouse experiments. First the *in vitro* transgenic plants that coming from rooting media (ME004-1) were carefully removed from the glass flasks and washed well with running tap water. To prevent bacteria and fungi contamination, the roots and lower leaves were cut with scissors. The same tubes that hold the vitro plants with the rooting media were washed to remove the remaining agar. Finally the cleansed vitro plants were put again inside the tubes, which contained 10-20 ml of HS enriched with NO_3^- as the N form and combined with Banrot® (5 mg/l). During the first five days, the tubes were covered with a perforated transparent plastic bag to allow the air exchange. The vitro plants were kept under HS during 22-30 days with a temperature of 28 ± 2°C and a photoperiod of 16 hours lightness and 8 hours darkness, the replacement of the HS was done every 4 days. After the period of growth under HS, the vitro plants were transplanted directly into sterilized soil at biosafety greenhouse conditions and covered during three days with a transparent plastic glass to avoid dehydration. The survival rate was recorded 35 DAT as the number of plants survived divided by number of transplanted plants.

Multiplication protocol of transgenic events using NO_3^- HS at greenhouse

In addition to the acclimation pipeline of *in vitro* plants from transformation lab, we have also developed protocol to multiply the transgenic events that were already established in the biosafety greenhouse. Stem cuttings (5 cm) from young cassava (3 months old seedlings) transgenic lines were incised carefully and hardened (NO_3^- HS) using floating hydroponic system described in above section. The HS was maintained at a pH of 6.5 and replaced every week. Three weeks after acclimation, plants were transplanted in the poly bags filled with sterile soil treated with insecticide (Lorsban® 10D) and fungicide (Banrot® 40WP) (Fig. 2-II). At 15 DAT, survival rate was recorded as the number of survived and dead plants.

Data Analysis

All statistical analyses were performed using the XLSTAT (2011) add-on for Microsoft Excel, with differences in mean values between lines evaluated using Student's t-test with Bonferroni's correction.

Results and Discussion

Effect of N forms on cassava hydroponic system and field survival

The acclimation of *in vitro* raised plantlets is essential for better survival and rapid establishment. The direct transfer of *in vitro* derived cassava plants to pot under greenhouse or field environments is not possible before a period of acclimatization; otherwise there is a high rate of mortality, due to regeneration in a cosseted environment with a very high humidity, varied light and temperature (Ospina et al. 2007, Cuesta et al. 2010). Direct transfer to a soil environment under greenhouse / field conditions also causes charring of leaves and wilting of the plants due to transplanting shock. The survival percentage is determined by the acclimation of the plantlets. It is therefore necessary to acclimatize the delicate cassava plants to strengthen the root and shoot portions. Several studies have also reported a positive correlation between transplanting performance of plant species and plant N concentration (Villar-Salvador et al. 2004; Oliet et al. 2009; Puértolas et al. 2011). This indicates that seedling nutrient composition ~~can~~ play an important role in the transplanting performance (Salifu and Timmer 2003), probably due to remobilization of internal N reserves (Millard et al. 2001; Silla and Escudero 2003). In the present study, hydroponic solution enriched with different forms of N were used as acclimation solution to adapt the *in vitro* plants under laboratory and greenhouse ~~level~~ conditions.

Aladele and Kuta (2008) reported that environmental and genotypic effects on the cassava growth rate were found in *in vitro* conditions. In ~~this~~ present study, significant variation ($P<0.05$) was observed among the studied genotypes ~~and~~ N sources in HS ~~conditions~~ under greenhouse (Table 1; Fig. 3). However, the trend of response in growth parameter as FW, MRL and RT showed high correlation ($0.790 \leqq R \leqq 0.997$) among all genotypes except that of GM4512-5. During the five weeks of acclimation, most of the varieties showed good adaptation to NO_3^- ~~and~~ NH_4NO_3 treatments. In contrast, under water and NH_4^+ treatments, the survival rate of genotypes showed 49.15 % and 49.27 %, respectively (Table 1). Even widely used cassava model cultivar

TMS60444 showed only 72.72 % of survival rate under water treatment. Compare to other routinely used methods (Chavarriaga-Aguirre et al. 2016), our hydroponic acclamation method improved 23- 50 % higher survival rate (Table 1). GM4512-5 showed high mortality during the acclimatization process, especially in treatments with H_2O and NH_4^+ (Table 1). In the first two weeks after HS acclimation, no significant variation was observed between treatments. After three weeks, however, it started to show significant differences in FW, RML and RT. NO_3^- and NH_4NO_3 were found to be superior in terms of FW and RML compared to NH_4^+ and water treatments (Fig. 4). At end of the acclimation (five weeks after transfer), we observed a huge difference among the N sources treatments (Table 2; Fig. 3). FW was found to be the most sensitive trait and it varied in response to each N source (Fig. 4). In NH_4^+ and water treatment, plant growth markedly stopped, and the inhibitory effect was even more dramatic under NH_4^+ conditions and N deficiency, respectively. Under NO_3^- and NH_4NO_3 treatments, FW was more than double compared to the other treatments. MRL was inhibited strongly under NH_4^+ conditions and root thickness was not increased under water conditions (Fig. 4). TMS60444 showed significantly (P<0.05) shorter MRL than SM3770-12 in response to NH_4^+, and significantly (P<0.05) thinner RT than GM5205-36 in response to NH_4^+. These results suggest that TMS60444 is a sensitive genotype with response to NH_4^+ (Table 2). N concentration in leaves of hardened plants did not show significant difference among the N sources (N concentration rate (mg/kg), 35.83±2.79, 32.88±1.74 and 33.67±1.57 at NH_4^+, NO_3^- and NH_4NO_3 HS, respectively). N is mainly taken up as NH_4^+ and NO_3^- by vascular plants and the N form available may be important to the distribution of species. Our results suggest that NO_3^- is a more important source to enhance the cassava growth under hydroponic conditions, while NH_4^+ is toxic for their growth. ~~Although~~ Although NH_4^+ is combined with NO_3^- (50:50), the toxic effect observed when NH_4^+ is provided as the sole N form is alleviated. In other hydroponic experiments, acid tolerant species were indifferent or grew best in a mixture of both N forms (Rorison 1985; Blacquière et al. 1988; Falkengren-Grerup and Lakkenborg-Kristensen 1994).

Survival rate of transplanting in the field was shown in Table 3. Significant variation (ANOVA; P < 0.05) was observed among genotypes, N sources, treatments and their interactions (Fig.5 and Table 2). As expected, TMS60444 showed higher field survival rate compared to other tested genotypes, indicating this genotype can be easily hardened through this system (Table 3). About treatment effect on survival rate, NO_3^- and NH_4NO_3 treatments showed a higher survival rate

(98.03% and 85.71%, respectively; Table 3) than NH_4^+ and water treatments (82.35% and 75.86%, respectively; Table 3). Even when the survival rate was not low in NH_4^+ and H_2O treatments, we found in NO_3^- and NH_4NO_3 hardened plants were much healthier and vigorous than other treatments by visual observation (Fig. 3 and 5). In conclusion, NO_3^- treatments were found to be the best in terms of growth and survival. We used NO_3^- for further validation.

Validation of NO_3^- acclimation system at laboratory level

The success of transgenic line development is not only based on robust transformation protocol, it also depends on the further acclimation and quick multiplication. Recent progress in cassava transformation has allowed the robust production of transgenic cassava even under suboptimal plant tissue culture conditions. The transformation protocol has so far been used mostly for the cassava model cultivar TMS60444 because of its good regeneration capacity of embryogenic tissues (Zainuddin et al. 2012). However, for deployment and adoption of transgenic cassava in the field, it is important to develop robust transformation and acclimation systems for farmer- and industry-preferred landraces and varieties. Since generating cassava transgenic lines involves cost and tedious procedures, the efforts and time of laboratory work is mainly based on the further quick acclimation and multiplication system. Current available methods involve delicate procedures to transfer transgenic lines directly to soil conditions without any pre acclimation (Jorge et al. 2000). Here, we introduced the NO_3^- acclimation system to speed up the establishment process at greenhouse conditions with high level of success (Fig. 6). By using this system, we found root development within four days after adaptation, which normally takes several weeks using the normal routine media method. In this study, survival rate after laboratory NO_3^- acclimation was around 90.3 % (112 of 124). The survived 112 plants were further transplanted into soil conditions under biosafety greenhouse conditions and showed a survival rate of 96.42 % (108 of 112). Ultimately, the final rate was around 87.09 % (108 of 124), which is quite high compared to the normal routine method (Jorge 1996, 2000). It was reported that assimilation of NO_3^- locally at its site of uptake leads to an increased influx of photosynthate and/or auxin which then stimulates lateral root growth in that region (Sattelmacher et al. 1993).

In the other experiment, we also transplanted several NO_3^- hardened transgenic lines to a confined field from N acclimation HS and we observed a 100% (89 of 89) survival rate which is very useful to cassava transgenic product development pipeline.

Rapid multiplication at greenhouse using stem cuttings

Despite the importance of this crop, it faces many problems, one of which is its low multiplication ratio (Osipina et al. 2007). Rapid multiplication systems can help overcome this hurdle. Transgenic technology testing is needed to generate a sufficient number of plantlets for each independent event for controlled environment testing. In this study, we also tested the stem cuttings of young transgenic cassava for acclimation and rooting using our developed hydroponic system with NO_3^- acclimation which showed a positive result (Fig 2-II). Totally, 89 of 92 stem cuttings (including both transgenic and non-transgenic) were hardened and multiplied under a biosafety greenhouse (survival rate of 96.7%) with minimum of six weeks and planted again soil conditions on pots for further use (Fig.2-II).

Advantage of floating hydroponic system on rapid cassava acclamation and acclimation

To our knowledge, a N hydroponic acclimation system to increase survivability of *in vitro* cassava plants have not previously been reported. As compared to other methods described in the literature (~~Santana et al. 2009~~; Carretero et al. 2009), one of its unique advantages is that it is very simple, cheap and speed up growth to multiply several *in vitro* plants in the short period (up to six weeks) of time under greenhouse and field conditions. Currently used acclamation methods are almost taking six months (Chavarriaga-Aguirre et al. 2016) to move the *in vitro* plants from greenhouse to field.

The plant survival rate showed in this study was also much higher than previous reports (~~Santana et al. 2009~~; Carretero et al. 2009; Koehorst-van Putten et al. 2012). Koehorst-van Putten et al. (2012) reported that 29 % of *in vitro* propagated cassava did not produce any roots, and 8 % of rooted cassava did not survive in greenhouse conditions. That means 37% of *in vitro* cassava died before field transplanting occurred. The other protocols developed by International Institute of Tropical Agriculture (IITA) and CIAT in Mozambique (Jorge 1996,) and Zimbabwe (Jorge et al. 2000), also reported a low survival rate (35 %) of cassava tissue culture plants in the transfer

to greenhouse conditions. Even using the other popular propagation methods like RATI and Temporary Immersion System, the percentage of losses are still very high (between 50 % and 95 %) (Ospina et al. 2007). Previous methods would leave the plants in greenhouse conditions for around 90 days before taking them to the field. However, our developed system allows us to transplant 35 days after acclimation in addition to the higher field survival rate. The new one-step N acclimation proves to be a very simple and efficient alternative to routine conventional methods available so far.

Future applications and Conclusions

In order to increase growth rate and avoid fungi and bacteria contamination, we suggest some minor modifications like adding an aeration system, covering the surface of the HS with a polyethylene foam. Since our developed HS system is simple and cost effective, this can be easily adapted by the laboratories located in the developing countries like Africa and South East Asia (Ng et al. 1992, Zok 1992; Mabanza et al. 1994). In the future, the application of hydroponic system may help to accelerate the cassava seed system and transgenic technology product pipeline. We have a confidence to modify the current hydroponic to aeroponic root platform in order to monitor cassava storage root development real time. In addition, our developed system may also be helpful for root physiologists and phytopathologists to design cassava hydroponic experiments to study nutrient and/or phytoplasma interactions.

Authors' contributions

MGS, SO and OC designed the study. OC, SO and AM implemented the experiments. OC and SO performed the statistical analysis. OC, SO and MGS drafted the manuscript. All authors read and approved the final manuscript. This work was performed in partial fulfillment of the requirements for the master degree of Mr.Oscar Castañeda-Méndez under the guidance of Dr. Michael Gomez Selvaraj, CIAT.

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Figures

Figure 1. *In vitro* micro-propagation of cassava.
A. Stock plantlets, B. SAM dissection, C. Sub-culturing in rooting media, D. Plantlets are allowed to growth. E. And about 6-7 cm long plants are ready to move to greenhouse.

Figure 2. Work flow of developed N acclimation hydroponic system
Acclimation of the *in vitro* cassava plants through HS (I). Rapid multiplication of stem cutting in the greenhouse using HS (II).

Figure 3. Effect of N source on cassava growth under HS. Phenotypic response of different genotypes A) TMS60444, B) SM1219-9, C) SM3770-12 after 35 days N acclimation under different treatments (H_2O, NH_4^+, NO_3^- and NH_4NO_3)

Figure 4. Time courses of growth rate on fresh weight, maximum root length and root thickness under different N sources
Each line graph shows mean ± SE (n = 8–14), Each graph line with square, diamond, circle and triangle showed mean data from H_2O, NH_4^+, NO_3^- and NH_4NO_3 treatments.

Figure 5. Field performance of direct transplanted *in vitro* cassava (TMS60444). Photo showing the effect of different treatments: A. H_2O. B. NH_4^+. C. NO_3^- .C. NH_4NO_3

Figure 6. Work flow of developed *in vitro* N hydroponic acclimation system at lab level

Photos showing (A) Gene transformed plants in the tissue culture media; (B) N Hardening of the *in vitro* cassava plants in the lab; (C) Rapid root and shoot development in HS at lab level; (D) transplanting of N hardened in the greenhouse.

Tables

Table 1. Effect of N source on cassava survival rate under greenhouse conditions

Table 2. Plant growth parameters with different N sources under HS

Table 3. Effect of N source on cassava survival rate under field conditions

Supplemental Table S1. Final nutrient concentration used HS treatments

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Figure

Fig.1 General ~~micro-propagation~~ of *in vitro* cassava plantlets.

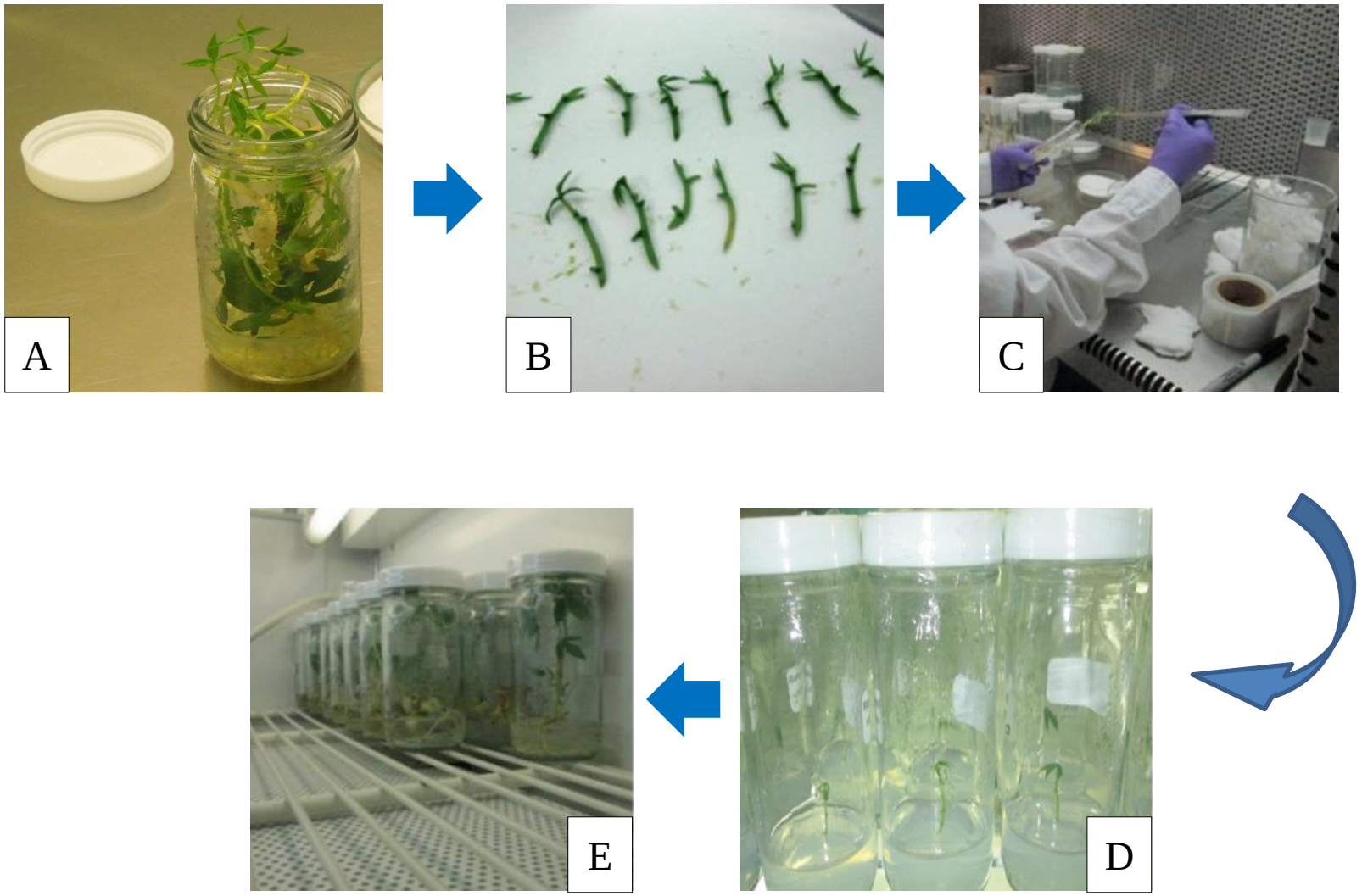


Fig.1

Fig. 2 N acclimation in HS in greenhouse conditions

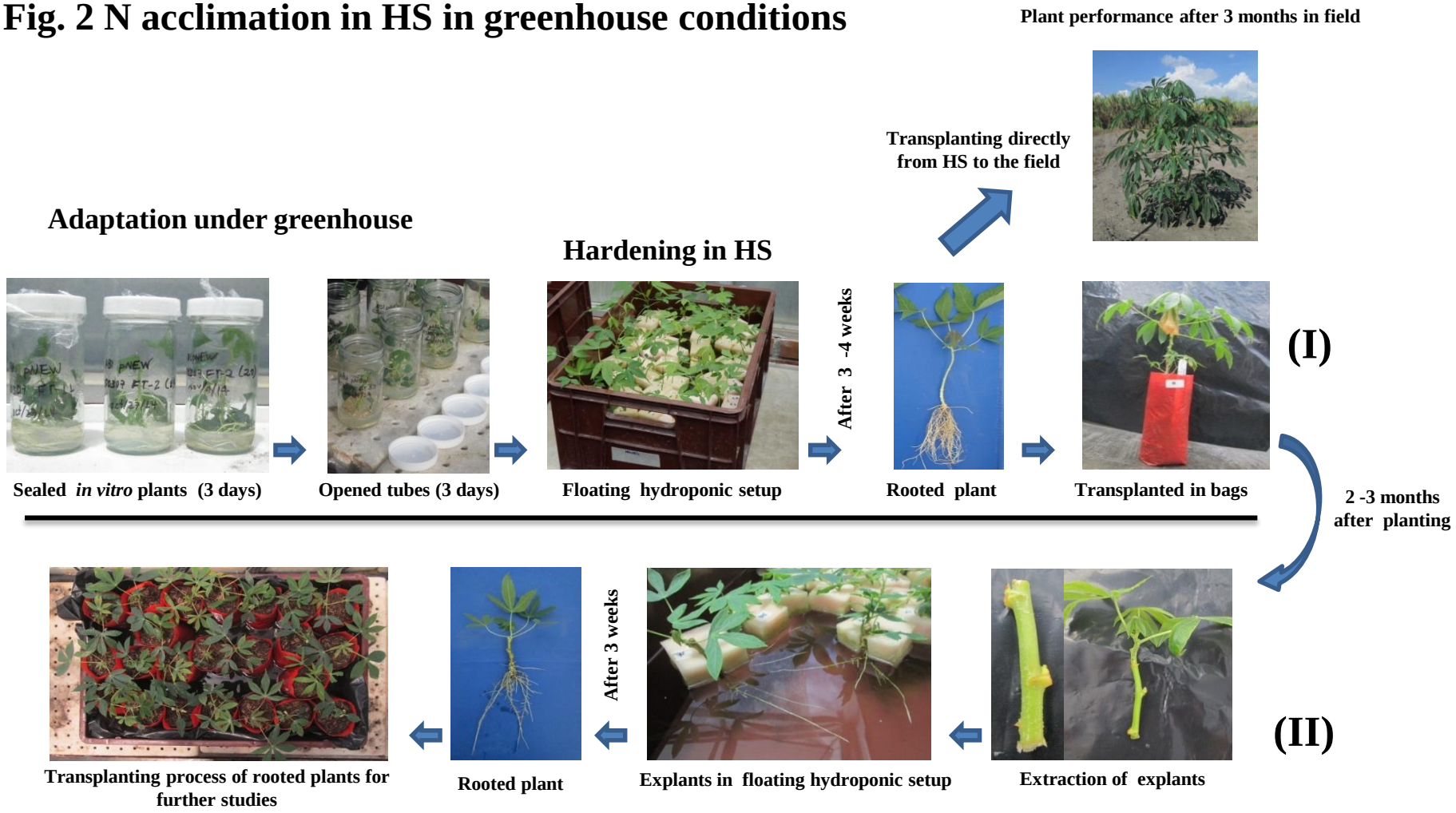


Fig.2

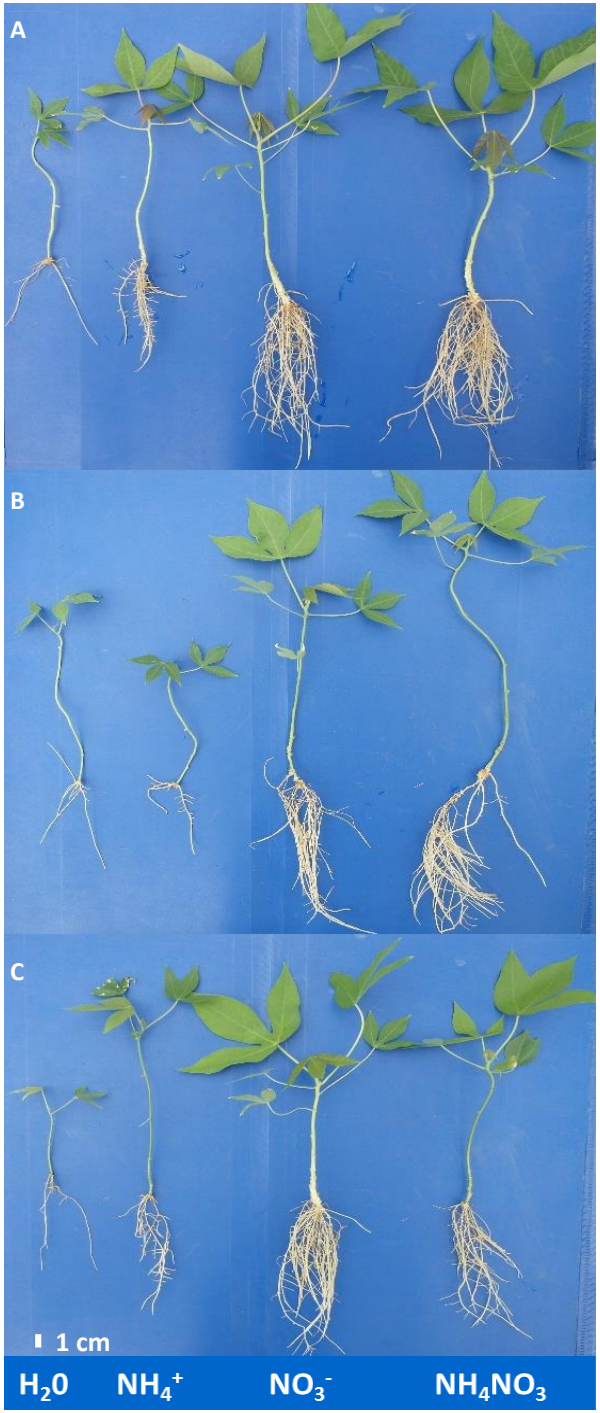


Fig.3

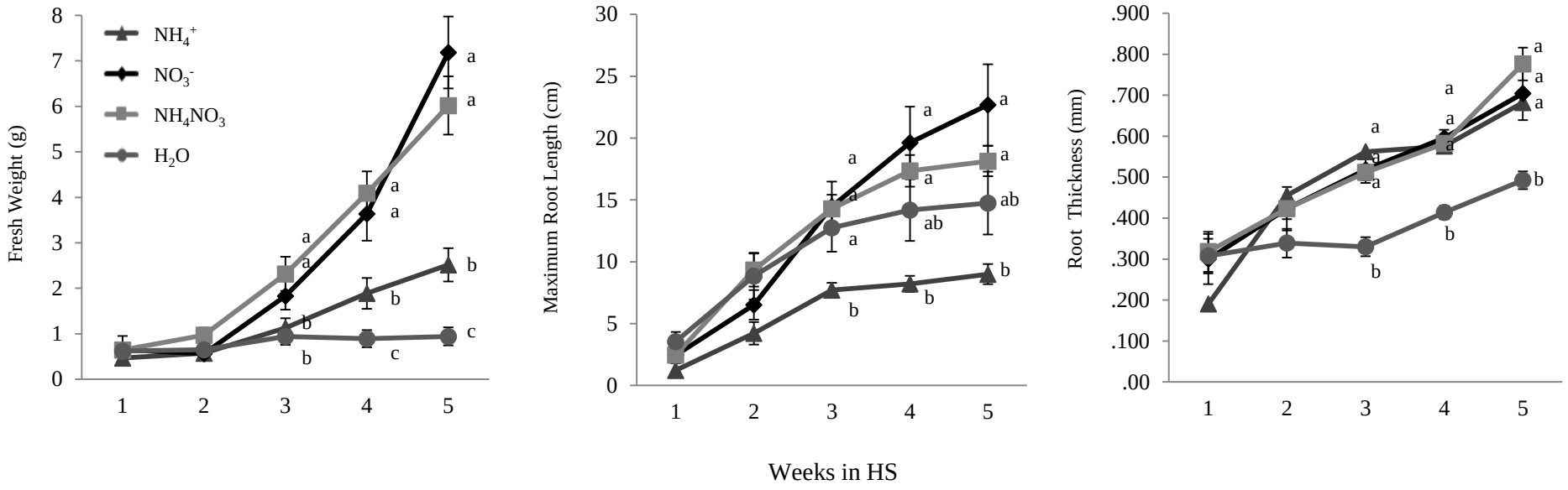

Fresh Weight (g)
NH_4^+
NO_3^-
NH_4NO_3
H_2O
Maximum Root Length (cm)
Root Thickness (mm)
Weeks in HS

Fig. 4

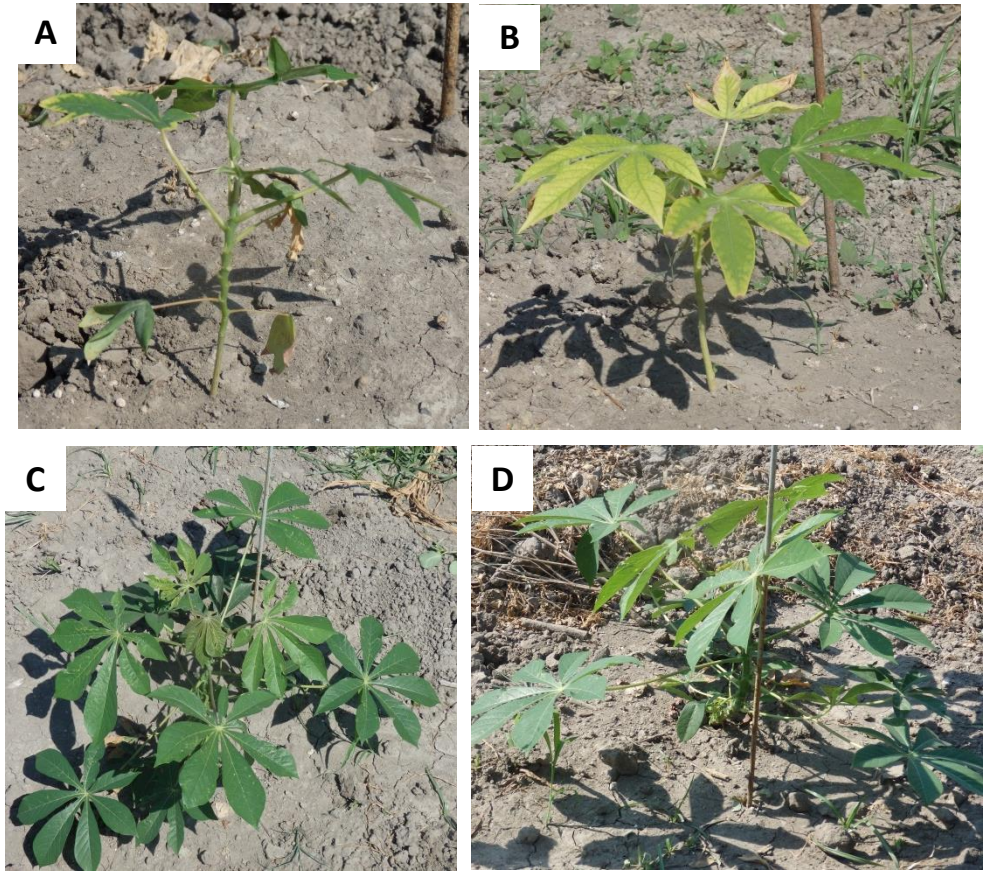
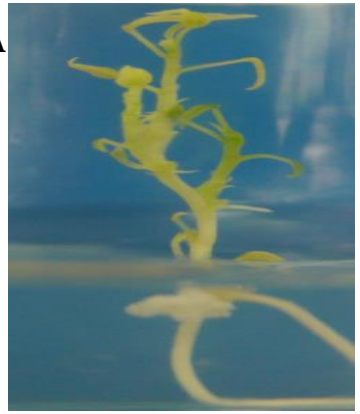


Fig.5

A



Gene Transformed plant in the soild media

B

Transfering plants in to the hydroponic system

C

Rapid root and shoot development after 5 and 21 days respectively

D

Transplanting in poly bags/pots for experiment or multiplication

Fig.6

Table

Table 1. Effect of N source on cassava survival rate (%) under greenhouse conditions

Treatment	TM60444	SM1219-9	SM3770-12	GM5205-36	GM4512-5	Total
H_2O	72.72% (8/11)	36.36% (4/11)	70% (7/10)	76.92% (10/13)	0 % (0/14)	49.15% (29/59)
NH_4^+	75% (9/12)	57.14% (8/14)	46.15% (6/13)	53.33% (8/15)	18.75 % (3/16)	49.27% (34/69)
NO_3^-	100% (11/11)	86.66% (13/15)	83.33% (10/12)	93.33% (14/15)	20 % (3/15)	75% (51/68)
NH_4NO_3	100% (11/11)	85.71% (12/14)	69.23% (9/13)	100% (15/15)	60 % (9/15)	82.35% (56/68)
Total	88.63% (39/44)	68.51% (37/54)	66.66% (32/48)	81.03% (47/58)	25% (15/60)	64.39% (170/264)

Number between brackets indicates (survived plants / evaluated plants)

Table 2. Plant growth parameters with different N sources under HS

Trait	Treatment	Genotype				
		TMS60444	SM1219-9	SM3770-12	GM5205-36	GM4512-5
FW (g)	H_2O	0.94 ± 0.20	0.79 ± 0.14	0.44 ± 0.10	0.71 ± 0.10	N.D.
	NH_4^+	2.52 ± 0.37	2.69 ± 0.69	3.12 ± 0.75	2.98 ± 061	1.19 ± 0.21
	NO_3^-	7.19 ± 0.79	8.11 ± 1.54	6.22 ± 0.87	4.99 ± 0.95	2.18 ± 0.72
	NH_4NO_3	6.02 ± 0.64	7.36 ± 1.05	6.58 ± 1.26	7.53 ± 1.25	5.21 ± 0.56
MRL (cm)	H_2O	14.75 ± 2.54	16.13 ± 2.57	8.29 ± 2.22	13.45 ± 1.42	N.D.
	NH_4^+	9.00 ± 0.81	12.06 ± 1.12	13.17 ± 1.34	10.83 ± 0.89	8.33 ± 1.48
	NO_3^-	22.68 ± 3.30	34.35 ± 3.28	27.35 ± 4.14	26.71 ± 3.44	21.33 ± 2.52
	NH_4NO_3	18.14 ± 1.23	23.13 ± 1.97	20.61 ± 2.71	19.83 ± 2.38	21.93 ± 1.68
RT (mm)	H_2O	0.49 ± 0.02	0.65 ± 0.07	0.56 ± 0.06	0.63 ± 0.04	N.D.
	NH_4^+	0.68 ± 0.04	0.80 ±0.04	0.84 ± 0.05	0.99 ± 0.10	0.77 ± 0.08
	NO_3^-	0.70 ± 0.07	0.72 ±0.05	0.72 ± 0.05	0.79 ± 0.04	0.68 ± 0.06
	NH_4NO_3	0.78 ± 0.04	0.83 ± 0.05	0.76 ± 0.04	0.85 ± 0.04	0.74 ± 0.02

Values represent the mean ± SE (n = 8–14)

FW: Fresh weight, MRL: Maximum root length, RT: Root thickness

Table 3. Effect of N source on cassava survival rate (%) under field conditions

Treatment	TM60444	SM1219-9	SM3770-12	GM5205-36	GM4512-5	Total
H_2O	100% (8/8)	75% (3/4)	57.14% (4/7)	70% (7/10)	N.D.	75.86% (22/29)
NH_4^+	77.77% (7/9)	75% (6/8)	83.33% (5/6)	87.5% (7/8)	100% (3/3)	82.35% (28/34)
NO_3^-	100% (11/11)	92.85% (13/13)	100% (10/10)	92.85% (13/14)	100% (3/3)	98.03% (50/51)
NH_4NO_3	90.90% (10/11)	83.33% (10/12)	100% (9/9)	80% (12/15)	77.77% (7/9)	85.71% (48/56)
Total	92.30% (36/39)	86.48% (32/37)	87.5% (28/32)	82.97% (39/47)	86.66% (13/15)	87.05% (148/170)

Number between brackets indicates (survived plants / evaluated plants)

Supplementary Table S1. Final nutrient concentration used in all HS treatments

Chemical Compounds	NH_4^+ -N HS (μM)	NO_3^- -N HS (μM)	NH_4NO_3 HS (μM)
Amonium Sulfate ($(NH_4)_2SO_4$)	600	-	300
Potassium Sulfate (K_2SO_4)	300	-	150
Potassium Nitrate (KNO_3)	-	600	300
Sodium Phosphate (Na_2HPO_4)	211	211	211
Calcium Chloride Dehydrate ($CaCl_2$ $2H_2O$)	340	340	340
Magnesium Sulfate Heptahydrate $MgSO_4$ $7H_2O$	446	446	446
EDTA Iron(III) sodium salt ($C_{10}H_{12}N_2NaFeO_8$)	54.4	54.4	54.4
Boric acid (H_3BO_3)	36	36	36
Manganese(II) Sulphate Monohydrate ($MnSO_4$ H_2O)	9.2	9.2	9.2
Zinc Sulfate Heptahydrate ($ZnSO_4$ $7H_2O$)	3	3	3
Cupric Sulfate Pentahydrate ($CuSO_4$ $5H_20$)	3	3	3
Sodium molybdate (Na_2MoO_4)	2	2	2

pH was adjusted to 6.5 by adding 1 N HCl

Supplementary Table S1. Final nutrient concentration used in all HS treatments

Chemical Compounds	g/ 100L (10 ppm)	NH_4^+ -N HS (μM)	NO_3^- -N HS (μM)	NH_4NO_3 HS (μM)
Amonium Sulfate ($(NH_4)_2SO_4$)	7.926/ - / 3.963	600	-	300
Potassium Sulfate (K_2SO_4)	5.229 / - / 2.615	300	-	150
Potassium Nitrate (KNO_3)	- / 12.132 / 6.066	-	600	300
Sodium Phosphate (Na_2HPO_4)	2.556	211	211	211
Calcium Chloride Dehydrate ($CaCl_2$ $2H_2O$)	5.293	340	340	340
Magnesium Sulfate Heptahydrate $MgSO_4$ $7H_2O$	11.388	446	446	446
EDTA Iron(III) sodium salt ($C_{10}H_{12}N_2NaFeO_8$)	1.652	54.4	54.4	54.4
Boric acid (H_3BO_3)	0.223	36	36	36
Manganese(II) Sulphate Monohydrate ($MnSO_4$ H_2O)	0.155	9.2	9.2	9.2
Zinc Sulfate Heptahydrate ($ZnSO_4$ $7H_2O$)	0.862	3	3	3
Cupric Sulfate Pentahydrate ($CuSO_4$ $5H_20$)	0.075	3	3	3
Sodium molybdate (Na_2MoO_4)	0.048	2	2	2

pH was adjusted to 6.5 by adding 1 N HCl

Authors' contributions

MGS, SO and OC designed the study. OC, SO and AM implemented the experiments. OC and SO performed the statistical analysis. OC, SO and MGS drafted the manuscript. All authors read and approved the final manuscript. This work was performed in partial fulfillment of the requirements for the master degree of Mr.Oscar Castañeda-Méndez under the guidance of Dr. Michael Gomez Selvaraj, CIAT.