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RESEARCH ARTICLE

Cassava (*Manihot esculenta*) Synthetic Seed Production for Germplasm Exchange

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The bulkiness of the planting material and its association with cassava mosaic disease are the major constrains for cassava germplasm exchange. Hence, an efficient technique for short-term conservation and exchange of its germplasm by encapsulating non-embryogenic propagules was standardised. Among the media tested for sprouting of *in vitro* sown synthetic seeds, the maximum sprouting (91.30%) and satisfactory growth of the plants was observed in a semi-solid MS basal medium with 3% sucrose. The regeneration potential of cassava synthetic seeds stored at room temperature (25±1^{o}C) after sealing aseptically in sterile polythene bags were studied. An average of 70.50% plantlets were recovered from the synthetic seeds stored for 42 days and sprouting was reduced drastically after 49 days of storage. The recovered plantlets from the synthetic seeds were acclimatised in planting trays comprising of autoclaved sand and garden soil (1:1). Three weeks after acclimatization 97.0% survival with normal plant growth was observed.

Key Words: Artificial seed, Encapsulation, Room-temperature storage, Short-term conservation, Tapioca

Introduction

Cassava (*Manihot esculenta* Crantz) is the fourth most important source of dietary energy in tropical regions after rice, sugar, maize and the ninth important crop in the world (Bokanga, 2002). Its cultivation and processing provides employment opportunities and ensure food security for millions of people in Africa, Asia and the Americas (FAO, 2020). Cassava based industry is immensely contributing to food security, poverty alleviation, economic growth and finally, rural development. This crop yields satisfactorily in low fertility soil and wastelands. Historically cassava was cultivated as a famine reserve crop as it was a more reliable alternative source of food during drought periods. Thus, cassava has recognition as a preference crop in the context of climate change adaptation strategies. Recently, this crop emerged as food and a profitable commercial crop of industrial significance (Aerni, 2006).

Cassava is commercially propagated vegetatively by the planting stem cuttings. This mode of propagation spreads pest and diseases particularly viral diseases and makes it difficult to conserve the germplasm. Because of stem cuttings' association with the spread of pests and diseases (Cassava mosaic disease-CMD), this mode of propagation also leads to quarantine problems during germplasm exchange. In addition, the rate of planting material multiplication in cassava is extremely low (1:8) and also stems do not store well for longer periods. Moreover, due to bulkiness of the stem cuttings (planting material), it is very difficult in handling and transport to distant places, thus restricting the spread of the crop in non-traditional areas and exchange of cassava genetic resources (Rajendran *et al*., 2005).

To maintain the diversity and sustainable genetic improvement of the crop, it is crucial to collect, conserve and utilize the available germplasm of a particular crop. An alternative means of short-term conservation and exchanging of cassava germplasm via synthetic seeds by encapsulating micropropagules is investigated. Synthetic seeds are artificially encapsulated micropropagules such as somatic embryos, shoot tips, nodes, axillary buds or any other tissues, capable of developing into a plant under suitable conditions, like a true seed. During handling and storage, plant propagule is protected by encapsulation against mechanical damage and drying. Hence, synseeds have practical applications for germplasm conservation and exchange (Hasan and Takagi, 1995). In addition, synthetic seeds are relatively inexpensive to produce and easy to handle during transport (Rao *et al*., 1998). In cassava, the synthetic seed can be produced by encapsulating nodal cuttings and shoot tips from *in vitro* raised plants using 3.0% sodium alginate and 100 mM calcium chloride (Hegde *et al*., 2016).

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Additional benefits of synthetic seeds include low production costs, short and long-term storability, facilitation of germplasm exchange between the laboratories, easy handling, transportation of propagules to distant places and subsequent propagation (Parveen and Shahzad, 2014). These synthetic seeds of cassava would allow direct sowing under *in vitro* upon receipt during the exchange of the genetic material between the researchers. In the present study, investigations have been undertaken to assess vaiability of synthetic seeds of cassava stored under room temperature, for facilitating exchange of encapsulated cassava propagules.

Materials and Methods

Nodal segments and shoot tips of popular cassava variety H226, measuring 2 to 5 mm were excised from 30 to 35 days old *in vitro* propagated plants on Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962). Protocol for synthetic seed production (Fig.1A) was similar as described by Hegde *et al*. (2016). The specially formulated standard purity chemicals used during the experiments were obtained from Hi-Media Laboratories Pvt. Ltd., India.

Sprouting and establishment of cassava synthetic seeds were studied under *in vitro* and *ex vitro* conditions with different media. For plant establishment from synthetic seeds under *in vitro* conditions, semi-solid as well as a liquid Hoagland (Hoagland and Arnon, 1950) and MS medium without as well as with 3.0% sucrose, were tested. The pH of the medium was adjusted to 5.8 and sterilization was done by autoclaving for 15 min at 121°C and 15 lb pressure. All cultures were incubated in the culture room at 25 ± 1°C under 16 h photoperiod provided by cool white LED lamps to observe the germination ability of the cassava synthetic seed in different media. For *ex vitro* germination, encapsulation solutions were supplemented with 0, 3, 6 and 9% sucrose with or without different concentrations and combinations of cytokinins (6-Benzylaminopurine – BAP @ 0.5, 1.0, 1.5 & 2.0 mg/L) and auxins (indole-3-butyric acid – IBA @ 0.5, 1.0, 1.5 & 2.0 mg/L) were tested. Synthetic seeds were sown *ex vitro* in pots containing different sterile (autoclaved) potting mixtures like cocopeat, vermiculite, sand with garden soil (1:1) and potting mixture readily available in the market (Agro-Bazar). These pots were covered with polythene bags to increase the relative humidity and kept in the nethouse for hardening.

Cassava synthetic seed storage study was conducted under room temperature and the germination ability of the stored synthetic seed was tested. Synthetic seeds were sealed aseptically under laminar flow in sterile, transparent and autoclavable polythene bags (HiDispo^{TM} Bag, HiMedia) (Fig.1B). These were stored at room temperature (25±1^{o}C) in dark up to 70 days. To study the germination ability, stored synthetic seeds were sown *in vitro* at seven days intervals on MS basal medium with 3% sucrose. The cassava plantlets derived from stored synthetic seeds were taken out from the medium after 42 days of incubation and washed with running tap water to remove the adhering medium. These plantlets were planted in protrays containing an autoclaved mixture of sand and garden soil (1:1) and acclimatised in a polyhouse after covering with perforated transparent polythene covers to maintain high relative humidity.

The experiments were carried out using a complete randomized design and repeated thrice with 25 synthetic seeds per treatment. Germination percentage, days taken for germination, number of shoots, shoot length (cm), number of leaves, number of roots and root length (cm) were recorded. The percent data was transformed using angular transformation before the analysis to test for statistical significance using OPSTAT (Sheoran *et al*., 1998).

Results and Discussion

In the present study, synthetic seeds of cassava variety H226 were produced by encapsulating nodal segments and shoot tips measuring 2 to 5 mm with 3.0% sodium alginate and 100 mM calcium chloride solutions with the complexing time fixed at 30 minutes. Encapsulation of explants with appropriate size, texture, stability and hardness is the key to producing synthetic seeds. Very hard beads restrict germination, while fragile beads make handling difficult. As reported by many researchers, 3% sodium alginate and 100 mM calcium chloride with 30 minutes complexing time is ideal for the production of synthetic seeds in several plant species (Ghanbarali *et al*. 2016; Baskaran *et al*., 2018; Kaminska *et al*., 2018 and Behera *et al*., 2020). *In vitro* produced somatic embryos are commonly used for the production of synthetic seeds since it behaves like a true botanical seed (Gantait *et al*., 2015). The rates of somatic embryo induction are very low in cassava and are highly dependant on the genotype, type of explant and growth regulators

concentration and combinations used (Anuradha *et al*., 2015). Production of synthetic seeds by encapsulating micro shoots with apical bud and nodes with axillary bud have been reported instead of somatic embryos (Gantait *et al*., 2015). Synthetic seeds produced by encapsulating these explants are economical and easier to handle as compared with somatic embryos. Hence, in the present work, cassava synthetic seeds were produced using nodal segments and shoot apices.

Effect of different semi-solid as well as liquid MS medium and Hoagland solution supplemented with or without 3.0% sucrose for the sprouting of synthetic seeds and establishment of complete cassava plant under *in vitro* condition is shown in Fig.1D-F. Sprouting of *in vitro* sown synthetic seeds were recorded when the encapsulation material splits open and the explants start growing. Among the treatments, maximum sprouting (91.30%) and growth was observed in semi-solid MS basal medium with 3% sucrose followed by liquid MS basal medium with 3% sucrose (90.48%). The difference in the sprouting of synthetic seeds between semi-solid and liquid MS basal medium supplemented with 3% sucrose was negligible. Drastic reduction in synthetic seed sprouting was observed in both liquid (42.86%) and semi-solid (57.14%) MS basal medium without sucrose. Similarly, 38.10% and 14.29% sprouting was recorded in Hoagland solution supplemented with or without sucrose, respectively (Fig. 2). Based on the observation, semi-solid MS basal medium supplemented with 3.0% sucrose is the best medium for the sprouting of cassava synthetic seed and establishment of the plant under *in vitro* condition. Similar to our results, high germination frequency of synthetic seeds was observed on full-strength MS medium in several plant species including *Solanum nigrum* (Verma *et al*., 2010), *Centella asiatica* (Prasad *et al*., 2014) and *Hedychium coronarium* (Behera *et al*., 2020).

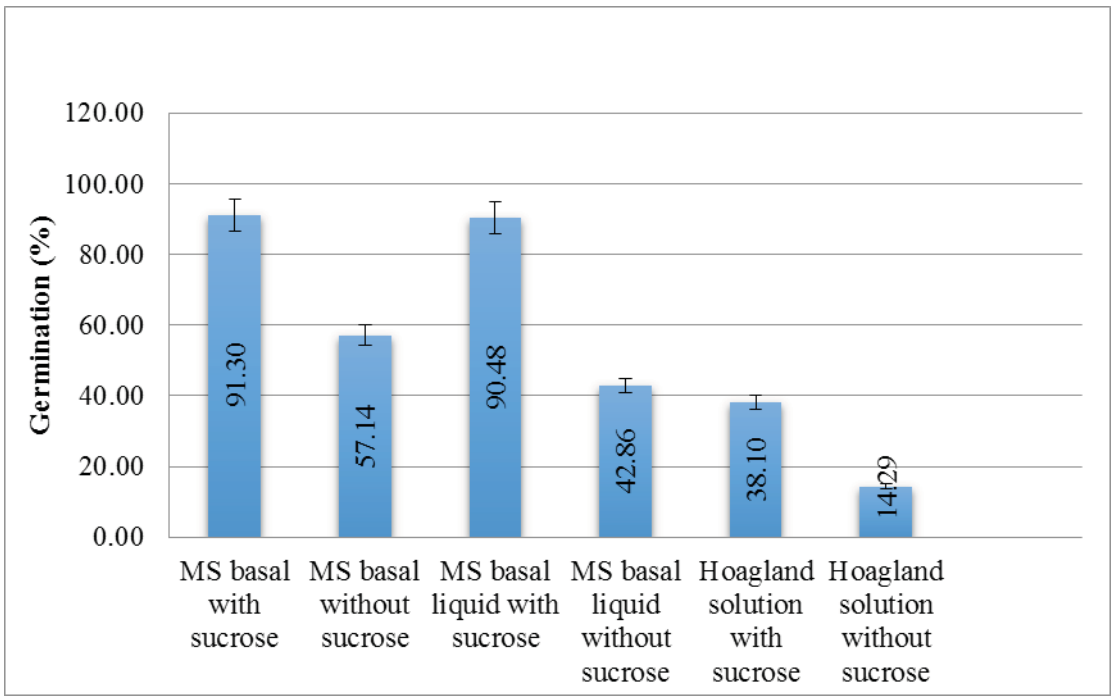
Synthetic seeds of the cassava in the present study failed to sprout and convert into plants under *ex vitro* conditions, despite supplementation of varied concentrations and combinations of sucrose and plant growth regulators to encapsulation solutions. Sterile potting mixtures including cocopeat, vermiculite, sand with garden soil (1:1) and potting mixture readily available in the market were unable to support the cassava synthetic seeds to produce a complete plant. Probably the nutrients in the micro-propagule as well as encapsulation material were not enough to support

Table 1. Germination of synthetic seeds of cassava after being stored at different durations under room temperature (25±1 ^{0}C)

Storage period (days)	Germination percentage, days after sowing								
	7	14	21	28	35	42	49	56	63
0	60.00 (7.80)	83.33 (9.18)	91.67 (9.63)	92.50 (9.67)	**92.50 (9.67)**	92.50 (9.67)	95.00 (9.80)	95.00 (9.80)	95.00 (9.80)
7	62.50 (7.91)	75.00 (8.72)	91.67 (9.63)	91.67 (9.63)	**91.67 (9.63)**	91.67 (9.63)	93.50 (9.72)	94.00 (9.75)	94.00 (9.75)
14	70.50 (8.45)	76.00 (8.78)	80.00 (9.00)	88.46 (9.46)	**88.46 (9.46)**	90.00 (9.54)	90.00 (9.54)	92.50 (9.67)	92.50 (9.67)
21	73.08 (8.61)	76.10 (8.78)	83.50 (9.19)	84.62 (9.25)	**85.00 (9.27)**	85.00 (9.27)	86.00 (9.33)	87.50 (9.41)	87.50 (9.41)
28	72.50 (8.56)	75.00 (8.71)	77.50 (8.85)	80.50 (9.02)	**83.50 (9.18)**	84.00 (9.21)	85.00 (9.26)	87.00 (9.37)	87.00 (9.37)
35	64.00 (8.06)	70.50 (8.45)	74.50 (8.68)	74.50 (8.68)	**75.50 (8.74)**	76.50 (8.80)	77.50 (8.85)	77.50 (8.85)	77.50 (8.85)
42	**55.50 (7.51)**	**61.50 (7.90)**	**65.00 (8.12)**	**67.50 (8.27)**	**70.50 (8.45)**	**70.50 (8.45)**	**74.50 (8.68)**	**74.50 (8.68)**	**74.50 (8.68)**
49	47.00 (6.93)	51.50 (7.24)	56.50 (7.58)	57.00 (7.61)	**58.00 (7.68)**	58.00 (7.68)	58.50 (7.71)	59.00 (7.75)	59.00 (7.75)
56	20.50 (4.64)	26.00 (5.19)	27.50 (5.34)	30.00 (5.57)	**30.00 (5.57)**	35.00 (5.99)	35.00 (5.99)	40.00 (6.40)	40.00 (6.40)
63	19.00 (4.47)	19.50 (4.53)	20.50 (4.64)	22.50 (4.85)	**22.50 (4.85)**	22.50 (4.85)	27.00 (5.29)	27.00 (5.29)	27.00 (5.29)
70	15.00 (4.00)	17.00 (4.24)	17.50 (4.30)	17.50 (4.30)	**21.50 (4.74)**	21.50 (4.74)	22.50 (4.85)	22.50 (4.85)	22.50 (4.85)
C.D.	0.426	0.445	0.458	0.464	**0.470**	0.472	0.471	0.474	0.474
SE (m)	0.144	0.151	0.155	0.157	**0.159**	0.160	0.160	0.161	0.161

(Figures in parenthesis are angular transformed values)

Fig. 1. Cassava synthetic seeds obtained by encapsulation of nodal segments and shoot tips (A); Cassava synthetic seeds sealed aseptically in sterile polythene cover for storage (B); Sprouting of cassava synthetic seeds during storage (C) and under *in vitro* (D); Cassava plant with well-developed shoot and roots obtained from synthetic seed (E&F); Acclimatized cassava plants with normal vegetative growth (G).

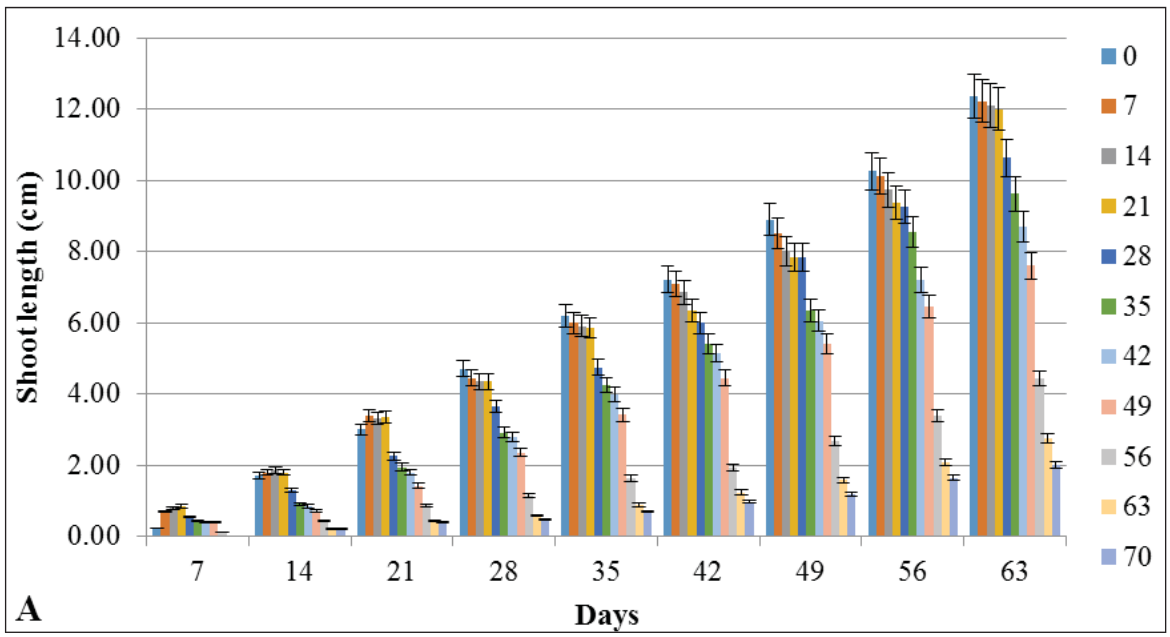


(Percentage error bar at 5%)

Fig. 2. Sprouting (germination) of cassava synthetic seeds on different culture media after 35 days of culture

the synthetic seeds for sprouting and establishment of plants under *ex vitro* conditions.

Sprouting percentage, shoot length and number of leaves were recorded at weekly intervals (Table 1, Fig. 3A & B). The highest sprouting/germination percentage (92.50%) was observed in freshly prepared cassava synthetic seeds (sown *in vitro* without storage). Sprouting decreased with increase in the duration of storage at room temperature. Drastic reduction of sprouting of encapsulated cassava explants was observed after 49 days of storage under room temperature. Stored synthetic seeds started sprouting after 14 days (Fig. 1C) and quick plant establishment was observed from them immediately after sowing *in vitro*. An average of 70.50% (35 days after



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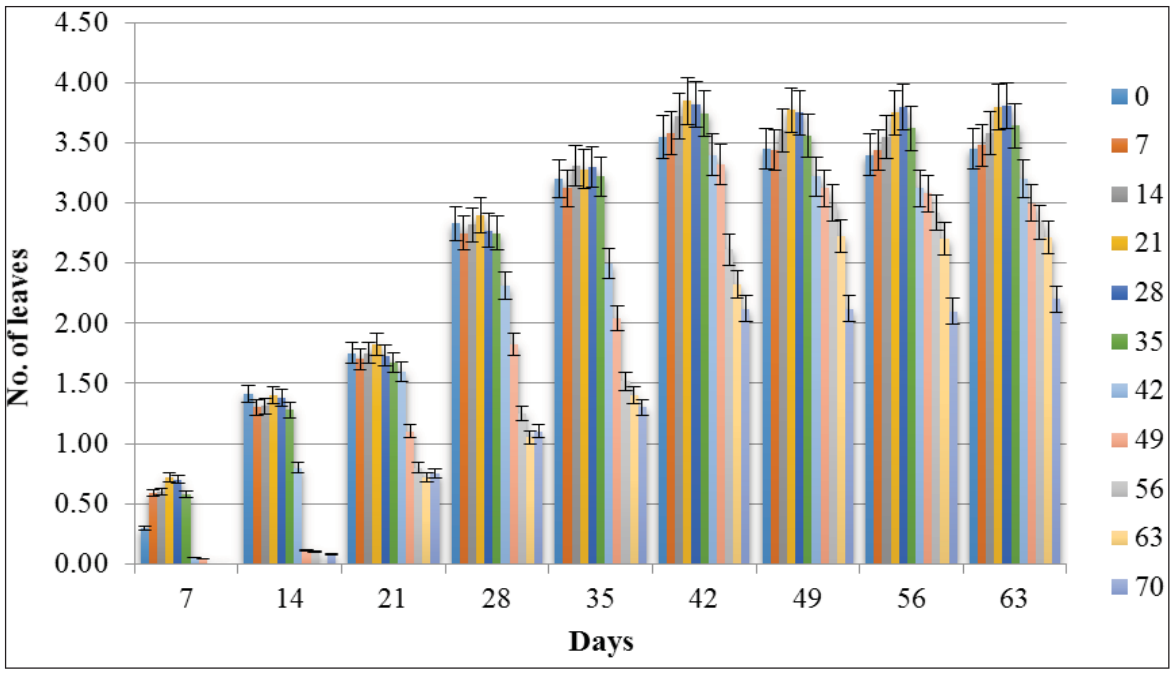

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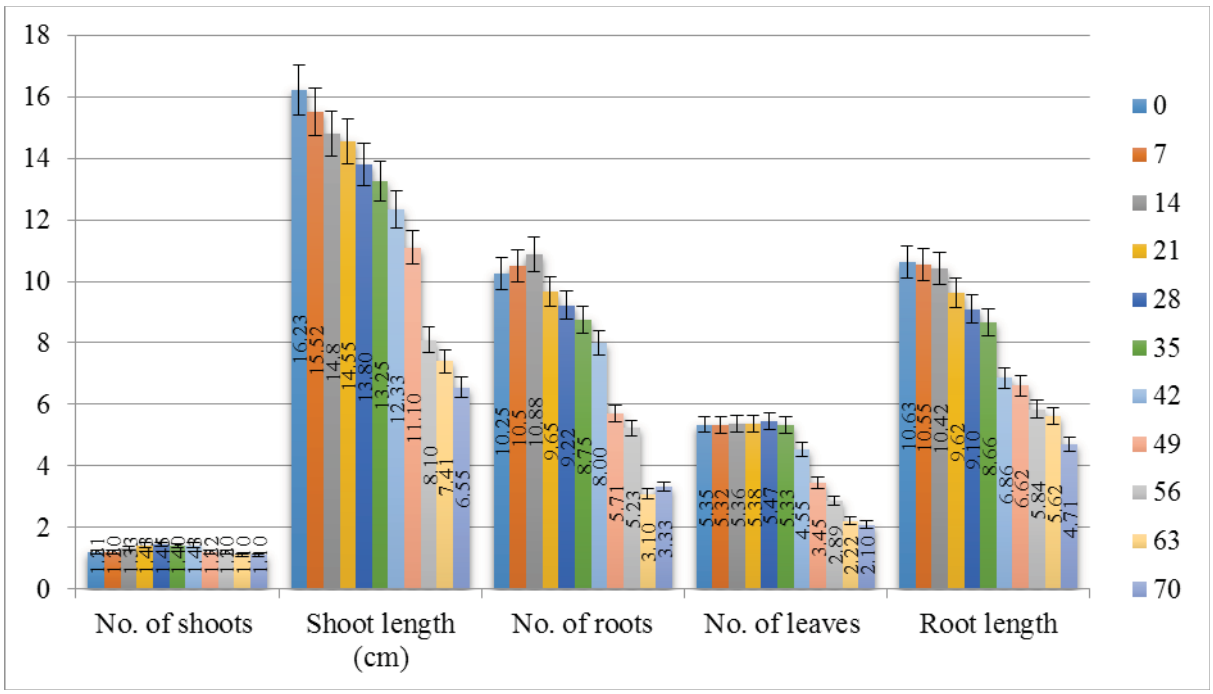
Fig. 3. Growth and development of *in vitro* cassava plants raised from synthetic seeds being stored at different durations under room temperature (A) Shoot length (B) Number of leaves

sowing *in vitro*) sprouting and optimum growth of the plants were observed from the cassava synthetic seeds stored for 42 days (Table 1, Fig. 3A & B). Significant difference was not observed for the average shoot length (cm) of *in vitro* plants obtained from stored synthetic seeds for different durations up to 42 days and it was reduced drastically beyond 42 days of storage (Fig. 3A). Average numbers of leaves were reduced among the *in vitro* plants obtained from the synthetic seeds stored for 49 days and beyond (Fig. 3B).

Cassava synthetic seeds stored at room temperature were sown *in vitro* at seven days intervals on MS basal medium supplemented with 3.0% sucrose. After 42 days of incubation under the culture room, cassava plants were taken out from the medium (Fig.1E & F) and observations were recorded. The average number of shoots ranged from 1.10 to 1.45 and no significant difference were observed between the treatments, while a significant difference was observed in shoot length (cm), number of roots, number of leaves and root length (cm). The average shoot length was highest (16.23 cm) among the plants raised from fresh synthetic seeds. It decreased significantly among plants obtained from stored synthetic seeds beyond 42 days. A similar trend was observed for root length and number of leaves, while the root number was maximum (10.88) among the plants raised from synthetic seeds stored for 14 days (Fig. 4). The short-term storage of synthetic seeds under room temperature (25±1 ºC) with acceptable plant recovery rate is reported in very few plant species including *Sphagneticola calendulacea* (Kundu *et al*., 2018), *Cineraria maritima* (Srivastava *et al*., 2009) and *Hedychium coronarium* (Behera *et al*., 2020).

Survival of plantlets was about 93.0% - 97.0% (data not given) after three weeks from the date of acclimatization and normal growth of the plants were observed (Fig. 1G). The recovered plantlets from the synthetic seeds acclimatised in *ex vitro* conditions with high (>90%) survival of plants is reported by several earlier researchers in many plant species (Srivastava *et al*., 2009; Kundu *et al*., 2018; Behera *et al*., 2020)

The acceptable frequency of plant conversion (70.50%) was observed from the cassava synthetic seeds produced by encapsulating *in vitro*-derived non-embryogenic micro-propagules like shoot tips and nodal cuttings, aseptically stored for 42 days under room temperature (Table 1). The technique described here is quick and simple. This protocol can be used as a novel delivery system for exchange of genetic materials between the laboratories or countries.



(Percentage error bar at 5%)

Fig. 4. Effect of short-term storage of cassava synthetic seed at room temperature on growth and development of *in vitro* plants (42 days after sowing)

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