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Research Article

Induction of micro-rhizomes for in vitro ginger (*Zingiber officinale* Rosco) disease-free planting materials regeneration

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ABSTRACT

Ginger has been a leading spice crop in Ethiopia until ginger bacterial wilt disease interrupted its production. The disease transmission is mainly through infected planting materials and products. Regeneration of healthy planting materials is among the best option to minimize the disease impact. This research aimed to induce and produce disease-free in vitro micro-rhizome in a combination of sucrose, BAP, ammonium nitrate, and silver nitrate. The results from observations indicated that sucrose and BAP were best combinations for induction of disease free micro-rhizomes. Ammonium nitrate and silver nitrate showed lower induction than sucrose and BAP combinations alone. The maximum viable number of micro-rhizome (5.67) and shoot number (10.33) were from 80.0 g/l sucrose and 6.0 mg/l BAP contained medium. Viability test indicated 80 % of the micro-rhizomes were sprouted after one month in soil. The micro-rhizome production potential observed in this experiment can enhance disease-free ginger production.

1. Introduction

Ginger (Zingiber officinale Rosco) is an important delicacy, medicinal, and spice monocotyledonous perennial herb belonging to the family Zingiberaceae. The cultivation and utilization of the crop in Ethiopia started during the 13th century when Arabs introduced it from India to East Africa [1]. Its production was mostly limited in the wetter regions of Southern Ethiopia. Ginger serves as a food flavoring, to strengthen and stimulate digestion, and as a medicinal plant in some cases [2]. The potential and importance of the crop is expressed by different conditions like total area covered and productivity per hectare of farmland. In terms of area harvested and total production, Ethiopia stood 10th and 14th respectively in 2011 among the 36 countries engaged in ginger production globally [3]. Later production and productivity of ginger significantly reduced to a minimal level due to wilt disease complex. In Ethiopia, economically important disease was not reported before 2012 production season for ginger. The only bottlenecks reported were a shortage of planting material for improved varieties, and poor field and post-harvest management practices [4]. The economic part rhizome serving also for ginger propagation requiring a large amount up to 2.2 tones to cover one-hectare farmland was another factor [5]. Moreover, during storage and cultivation, rhizomes used for vegetative propagation are susceptible to pathogens particularly fungus (Pythium) and bacteria (Ralstonia solanacearum) causing rhizome rot and bacterial wilt diseases respectively [6]. Bacterial wilt is now very important in Ethiopia mainly in major ginger-producing areas of South and South Western parts, where ginger has been widely produced for commercial purposes. Disease incidence in the field usually ranges from 10 to 40 % globally, but the disease also can destroy the crop with an incidence of 80 to 100 % in Ethiopia [7,8]. Regeneration of healthy planting materials is among the best options to reduce the disease impact [9]. Therefore, options to produce disease-free planting materials are vital. To this end, in vitro induction and production of disease free micro-rhizomes in modified Murashige and Skoog [10] (MS) growth media was conducted in designed experiment. Micro-rhizomes are miniature rhizomes that can be generated in vitro, stored and planted in pots or nursery beds conveniently for further multiplication [11]. In addition, micro-rhizomes have got enough potential to be used by commercial growers as disease-free planting material, produced in vitro irrespective of seasonal fluctuations, easily transferable, and sown like seeds [12]. Hence, this research was undertaken to enhance disease-free rhizome seed generation through in vitro micro-rhizome induction and production technique establishment.

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2. Materials and methods

2.1. Experimental materials

Ginger shoot tips of 'Volvo' cultivar; a popular farmer's variety was in vitro initiated using in vitro regeneration protocol previously optimized [13]. Plantlets from the multiplication stage are transferred to growth regulators free MS medium to get physiologically similar experimental materials. Then plantlets growing on growth regulators free medium were used as experimental materials as per treatments and design.

2.2. Stock solution and media preparation

Stock solution for MS medium was prepared by salt type category for all nutrients, growth regulators, vitamins, and organic additives and stored at 4 °C until use. Agar at 6.0 g/l of the medium was used to semi-solidify the media during preparation after the pH was adjusted to 5.8 for all treatments throughout the experiment.

2.3. Treatments and experimental design

The experiment had two phases. In the first phase, there were four different concentrations of sucrose (30, 60, 80, and 100 g/l) and three concentrations of benzyl amino purine (BAP) at 3.0, 6.0, and 9.0 mg/l MS media. The second phase combined ammonium nitrate (NH_4NO_3), and silver nitrate ($AgNO_3$) each consisting of three levels. Ginger micro-propagation medium; containing 30 g/l sucrose, 2.0 mg/l BAP, and 1.0 mg/l kinetin was included as a control in both phases. A completely randomized design (CRD) structured in a factorial arrangement resulted in 13 treatments for the first phase and 10 for the second phase. Each treatment was replicated five times using glass culture jars with 40 ml medium each contained five explants. The experiment at each stage was repeated three times for verification of treatment effects.

2.4. Culture condition

All required materials including growth media were sterilized at 121 °C temperature and 103.42 Kpa pressure for 20 min. Culture jars contained experimental materials were maintained in growth room with average of light intensity of 185.2 μmolm-2 s-1 (10,000 lux), a temperature of 27 ± 2 °C, and relative humidity of 80 % under a 16 h photoperiod. To get an optimum number of well-established micro-rhizomes per shoot, cultures were directly transferred once after six weeks of first culturing to a similar fresh medium.

2.5. Micro-rhizome viability test

Micro-rhizomes were collected from the base of shoots carefully and planted in plastic pots (Fig. 2a) filled with a sterile soil mix in a 2:1:1 ratio (topsoil, compost, and sand). Pots containing micro-rhizomes were then placed in a screen house for sprouting observation.

2.6. Data analysis

Data for the date of micro-rhizome induction, rate of micro-rhizome induction, number of micro-rhizome per shoot, weight of micro-rhizome per shoot/jar, number of shoots per explant, number of roots per explant was recorded. Days to sprouting and the number of rhizomes sprouted as a percentage was also recorded during in vivo viability testing. Data collected is subjected to analysis of variance (ANOVA) using the SAS version 9.3 [14]. Mean comparison among the treatments and significance test was computed using Fisher protected least significant difference test at 5 % probability. Linear correlation coefficient analysis to understand the association of in vitro shoot growth and micro-rhizome traits was done using Minitab 19.1.1 [15] at a 95 % confidence interval.

3. Results and discussion

3.1. Sucrose and BAP effect

The result for in vitro micro-rhizome induction by the combined effect of sucrose and BAP showed that there is a significant difference among treatments at a probability level of 5 % (Table 1). Shoot buds cultured in MS medium supplemented with 80 g/l sucrose and 6.0 mg/l BAP responded better in terms of most parameters recorded (Fig. 1a). One-way analysis of variance (ANOVA) also revealed highest amount (100 g/l) sucrose-containing medium has no significant effect on micro-rhizome induction and development (Table 1). On the other hand, the medium with 30 g/l sucrose with 3, 6, and 9 mg/l BAP did not show a significant difference from the control medium supplemented with 30 g sucrose and 2.0 mg/l BAP (Fig. 1b). The result indicated that BAP has less effect on micro-rhizome induction in ginger as compared to sucrose. Maximum single fresh micro-rhizome weight (3.92 g) with a mean of four active buds was from medium supplied with the 80 g/l sucrose with 6.0 mg/l BAP. The highest mean number of shoots (10.33) was also from the same medium. However, micro-rhizome induction was not observed on a medium containing 30 g/l sucrose at all levels of BAP from 2-9 mg/l including control medium which is a standard micro-propagation media for ginger in the experimental laboratory.

On medium containing the highest concentration of sucrose (100 g/l), induction was numerically better but their viability was poor due to low biomass and the limited number of active buds to sprout. Root growth was also highly affected due to treatment effects in which the maximum number and length of roots were recorded from a medium containing 60 g/l sucrose with 3, 6, and 9 mg/l BAP (Table 1) which might be at the expense of shoot and micro-rhizome development. Shoot growth and micro-rhizome induction trend showed that shoot number, shoot length, leaf number, root number, and length decreases as sucrose increases from 30 g to 100 g/l. While, the rate of micro-rhizome induction, micro-rhizome number, and total micro-rhizome fresh weight increased as the sucrose amount increases up to 100 g/l at all levels of BAP (Fig. 1a and b).

A previous study report [11] indicated that sucrose from 80 to 90 g/l with different types and concentrations of growth regulators has better-induced micro-rhizome in ginger after one month of culturing. Another study also revealed that an increase in sucrose decreases plantlet's number and height due to high bulging at the base than leaf and stem growth [16]. Our results in this experiment confirmed a higher level of sucrose (80 g/l) with an optimum level of BAP (6 mg/l) is better to induce and produce significant number of viable micro-rhizome per shoots is in agreement with the previous study findings. The inverse proportionality of shoot and root growth parameters to sucrose levels is due to the biological response of tissues to carbon level in the growth media as also reported by previous findings [16]. Another study indicated that MS medium fortified with elevated amount of BAP and sucrose than standard was better for the maximum number and fresh weight of micro-rhizome agrees to our findings [17]. Moreover, our experiment showed that BAP concentrations above 6 mg/l did not affect the micro-rhizome induction instead it affected root number and length at 9 mg/l combined with 60 g/l sucrose (Table 1). It was also reported that the negative effect of sucrose at elevated level (>100 g/l) makes plantlets etiolate and die [16]. The same author reported sucrose at medium level plays the main role in micro-rhizome induction and development. The low effect of BAP levels on micro-rhizome recorded here also further confirmed that sucrose mainly influenced explants to induce micro-rhizome than growth regulators (Table 1). The elevated amount of sucrose induces micro-rhizome by increasing carbohydrate storage and creating bulging at the base of shoots that later develops into viable rhizome. This was confirmed previously by nutrient composition analysis of ginger rhizome in which carbohydrate takes the largest proportion (12.3 %) next to 80 % water in the fresh rhizome and 50 % of dry ginger [18].

Table 1
Effect of sucrose and BAP on micro-rhizome induction and growth.

#Treat.	Combinations		Recorded parameters						
	Sucrose	BAP	MRN/S	NB/R	NS/E	SL(cm)	RN	RL(cm)	MRFW/S(g)
1	30	3	0.0	0.0	6.33	6.5	16.00	10.26	0.0
2	30	6	0.0	0.0	4.66	6.26	16.67	10.33	0.0
3	30	9	0.66	0.66	5.67	6.43	11.67	12.16	0.76
4	60	3	2.33	2.66	4.67	5.43	19.00	11.16	3.03
5	60	6	2.33	3.00	7.33	6.43	21.67	12.36	3.46
6	60	9	3.66	2.66	6.00	5.83	23.33	13.03	3.46
7	80	3	4.66	4.33	9.00	2.46	13.00	9.00	3.79
8	80	6	5.66	5.00	10.33	2.36	11.00	8.23	8.08
9	80	9	5.33	4.67	9.33	2.73	11.00	8.76	6.98
10	100	3	6.00	1.00	3.00	1.40	6.33	6.5	5.95
11	100	6	6.33	1.33	4.33	1.6	6.67	6.26	5.90
12	100	9	5.66	2.00	4.67	1.83	7.33	5.50	6.5
13	30	2	0.0	0.0	9.67	8.83	20.00	12.36	0.00
CV (%)			23.96	21.53	15.25	7.35	7.26	5.45	21.66
LSD (5 %)			1.73**	1.09*	1.65**	0.57*	1.75*	0.88*	1.32**

Note: MRN-micro-rhizome number per shoot, NB-number of node per rhizome, NS-number of the shoot, SL- shoot length, RN-root number, RL root length, and MRFW-micro-rhizome fresh weight

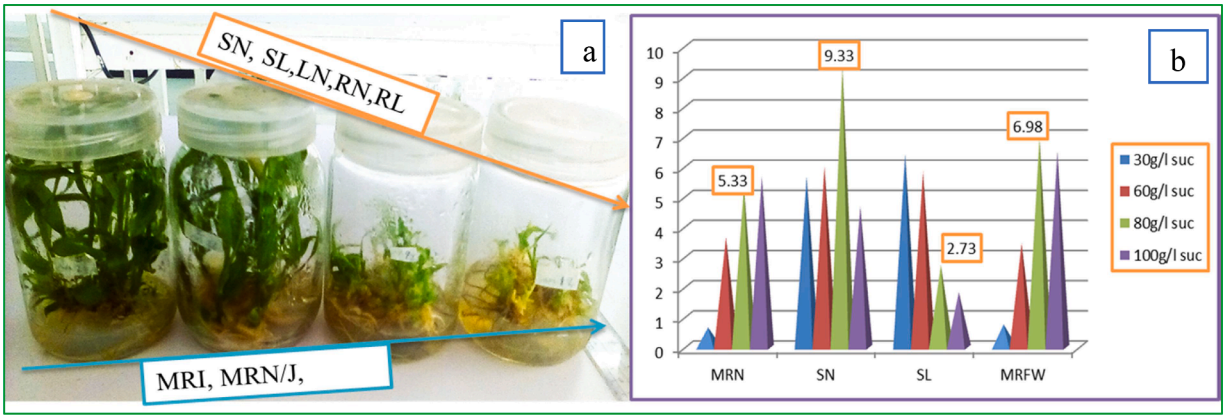

Fig. 1. Micro-rhizome induction and development on different combinations of sucrose and BAP. a. Rhizome growth on medium containing 6 mg/l BAP and 30, 60, 80 & 100 g/l sucrose (left to right). b. Graphic representation of shoot growth and micro-rhizome development on MS medium with 6 mg/l BAP and four levels of sucrose. Numbers in text box are micro-rhizome number (MRN), shoot number (SN), shoot length (SL/cm) and micro-rhizome fresh weight (g/shoot) at 80 g/l sucrose and 6 mg/l.

Exceptional maximum viable number of micro-rhizome (17), per jar and large number of shoots (23) were achieved simultaneously from a medium containing 80 g/l sucrose and 6 mg/l BAP. This result was obtained by direct transfer of induced micro-rhizome to the respective medium once after six weeks of culturing to take another six weeks in fresh media (Fig. 2a). From this, we have learned that the combination of sucrose and growth regulator and elevating time of culturing is crucial to produce a large number of viable micro-rhizome. The technique also resulted in more than double the number of shoots simultaneously from the same medium as compared to control micro-propagation media (Fig. 2b).

3.2. *Correlations among in vitro growth parameters*

Linear correlation analysis revealed the association of growth parameters that have a strong positive or negative significant correlation

Fig. 2. Induced micro-rhizome records. a. Micro-rhizome in vitro b. maximum number of micro-rhizome and shoots per jar achieved, c. Micro-rhizomes active buds to sprout and d. Single micro-rhizome fresh weight (3.92 g).

with micro-rhizome induction and growth. Micro-rhizome fresh weight has a negative significant linear correlation with shoot number, shoot length, root number, and root length (Table 2). Whereas, the fresh weight of rhizomes has a significant positive correlation with the number of micro-rhizome and the number of active buds per rhizome (Fig. 2c). Previous research results reported [17] also showed that shoot and root growth is inversely proportional to micro-rhizome number and weight. This might be due to plant cells physiological response as affected by available nutrients in the medium. In this case, the saturated amount of sucrose has promoted rhizome induction and growth rather than shoot and roots.

In the micro-rhizome sprouting test, rhizomes with a large number of active buds and good biomass have a high rate of sprouting up to 80 %. In contrast, those with low weight and less number of buds failed to sprout and grow to ginger plants in soil. Therefore, to achieve viable micro-rhizomes the number and growth should be optimum by using the appropriate combination of sucrose and growth regulators. The viability of micro-rhizomes with high fresh weight (>2 g) and at least three active buds was better as compared to those with less weight and a limited number of buds. Linear correlation coefficient at 95 % confidence interval (Table 2) revealed that micro-rhizome fresh weight, number of micro-rhizome, and active buds have a significant positive correlation with each other but weak and non-significant with others like shoot number and length as well as with root number and lengths (Table 2).

3.3. Effect of ammonium nitrate and silver nitrate

The second phase of the experiment involved ammonium nitrate (NH_4NO_3) and silver nitrate ($AgNO_3$) at different levels (Table 3) with and without sucrose and BAP at 80 g/l and 6 mg/l respectively. The results revealed that all NH_4NO_3 and $AgNO_3$ combinations have failed to result in better micro-rhizome induction and growth as compared to the medium supplied with 80 g/l sucrose and 6 mg/l BAP alone (Table 3). Analysis of variance indicated that means from a medium with 80 g/l sucrose and 6 mg/l BAP are highly significant at 5 % probability for the number of micro-rhizome and weight. Whereas, the lowest mean was from medium supplemented with 0.55 g/l NH_4NO_3 and 1.9 g/l $AgNO_3$ in contrast to 80 g/l sucrose and 6 mg/l BAP for the corresponding parameters. The result in Table 3 also revealed that maximum shoot and root growth was recorded from a control medium supplied with 30 g/l sucrose and 2 mg/l BAP as it was in the first phase (Table 1).

Recent research reports states that the maximum micro-rhizome weight on MS medium supplemented with low ammonium nitrate and high silver nitrate effect [19]. In spite of nearly similar amounts of sucrose and BAP used like in this experiment, the result is partially in contradiction with our findings. In this experiment, both low concentrations of ammonium nitrate and high concentration of silver nitrate have negatively affected micro-rhizome induction and mean fresh weight. Another research report on the other hand stated silver nitrate at a very small level has improved micro-rhizome and shoot multiplication but was inhibitory at a high concentration of around 2.9 g/l [20]. The same author elaborated that the small amount of silver nitrate enhances induction by inhibiting the ethylene effect whereas at high concentrations it affects nutrient uptake to explants and negatively affects in vitro growth. Poor media quality and inhibition of micro-rhizome and shoot growth might be due to the nature of silver nitrate. Low ammonium nitrate enhanced micro-rhizome development by limiting upper part growth due to low nitrogen in media that can serve for protein synthesis [19]. However, this has not significantly affected micro-rhizome induction in this experiment, which may be due to the genotype effect. Moreover, we observed media color change to orange at 2.9 g/l $AgNO_3$ that might be due to side reaction with other media components and an inhibition effect on explants resulting in the exudation of secondary metabolites (Fig. 3a).

Table 2
Linear correlation coefficient of in vitro growth parameters.

Traits	MRN/S	NB/R	NS/exp	SL(cm)	RN	RL(cm)
NN/R	0.612**					
NS/Exp	-0.042	0.546				
SL	-0.931**	-0.493	0.164			
RN	-0.641**	-0.065	0.247	0.813*		
RL	-0.751**	-0.150	0.267	0.889*	0.896*	
MRFW(g)	0.951**	0.711* *	0.096	-0.866**	-0.582*	-0.689**

Note: MRN/S- number of rhizome per shoot, NN/R- number of nodes per rhizome, NS/exp- number of shoots per explant, SL- shoot length, RN- root number and RL- root length.

3.4. Micro-rhizome viability

Rhizome sprouting ability was tested in sterilized soil mixture (2:1:1) topsoil, sand, and coffee husk respectively. After one month of planting, 80 % of micro-rhizome sprouted with vigorous growth from medium enriched by 80 g/l sucrose and 6 mg/l BAP (Fig. 4a) followed by 70 % from medium supplemented with 60 g/l sucrose and the same amount of BAP. On the other hand, the lowest percentage of planted micro-rhizome emerged from media containing 100 g/l sucrose and 9 mg/l BAP. The low viability of micro-rhizomes may be due to their low size and a small number of active buds declined them to emerge. Most of the studies on ginger micro-rhizome production have not included the performance of the in vitro-produced micro-rhizomes. Only one research reported micro-rhizomes ability of sprouting on moist sand after two weeks but did not revealed the rate of viability [11]. Micro-rhizome viability rate recorded here (80 %) can be considered as base for future works to maximize rate and improving micro-rhizome quality during induction and production.

4. Conclusion

These experiments conducted to induce and produce viable micro-rhizome ginger seed generally indicated that micro-rhizomes induced and produced on MS medium supplemented with sucrose (80 g/l) and 6 mg/BAP can be a source of disease-free planting materials. The finding of obtaining micro-rhizomes and plantlets at the same time is the first in its kind. The micro-rhizome induction protocol optimized can be used for large-scale clean seed production irrespective of season and for in vitro conservation of ginger germplasm. Repeated experiments with modification are future research areas to get the maximum number of micro-rhizome. Further work to increase the viability percentage under different in vivo growth conditions including open field will be a research area.

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Author's contribution

All authors have contributed to the planning and implementation of the work. Genene Gezahegn performed material preparation, data collection, analysis, and first draft. Tileye Feyissa and Yayis Rezene did technical support, supervision, and edition of previous versions. Hence, all authors read and approved the final manuscript for submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

Table 3
Effect of ammonium nitrate and silver nitrate on micro-rhizome induction.

#Treatment	Combinations		Measured in vitro traits					
	NH_4NO_3 (g)	$AgNO_3$ (g)	NR/Ex	RW (g)	NS	SL(cm)	RN	RL(cm)
1	1.65	0.9	2.00	2.50	6.33	4.7	11.00	3.03
2	0.825	0.9	2.33	1.70	3.33	4.67	15.00	3.20
3	0.55	0.9	1.67	0.60	3.33	1.93	4.67	4.03
4	1.65	1.9	0.67	1.77	3.00	2.83	5.00	1.87
5	0.825	1.9	0.35	0.70	3.67	3.97	8.00	1.90
6	0.55	1.9	0.33	0.33	2.00	2.90	3.33	1.97
7	1.65	0	5.67	6.00	9.00	5.83	11.33	6.63
8	0.825	0	4.00	3.67	5.67	3.27	14.33	2.70
9	0.55	0	2.33	1.43	1.67	1.07	2.67	1.50
10	1.65	0	0.33	0.23	10.33	8.73	18.33	11.43
CV (%)			22.26	20.21	11.95	9.10	8.23	19.91
LCD (5 %)			0.75**	0.65**	0.98**	0.62**	1.32**	1.23**

NR- number of micro-rhizome per shoot, RW- rhizome weight, SN- shoot number, SL- shoot length, RN- root number, RL- root length and control in use for ginger micro-propagation.

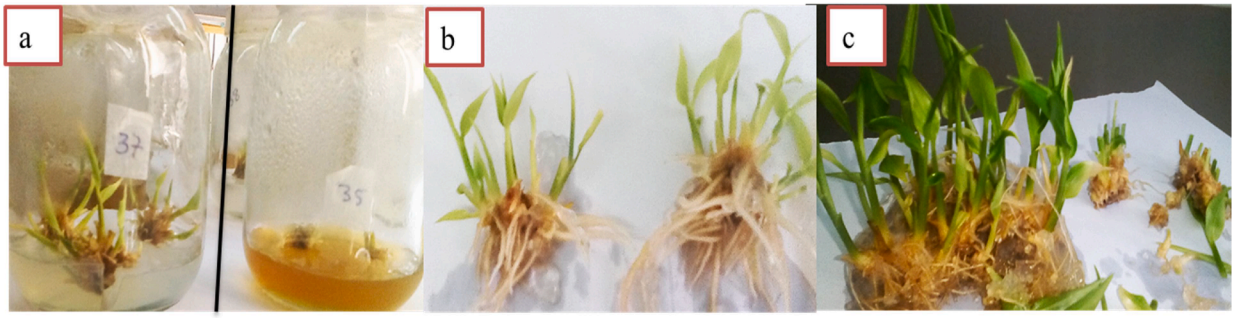

Fig. 3. Effect of silver nitrate and ammonium nitrate on micro-rhizome induction. a. media without silver nitrate (left side) and media with 2.9 g/l silver nitrate (right side), b. Plantlets from low ammonium nitrate and high silver nitrate with poor micro-rhizome growth, c. plantlets on medium containing sucrose and BAP alone with induced micro-rhizome.

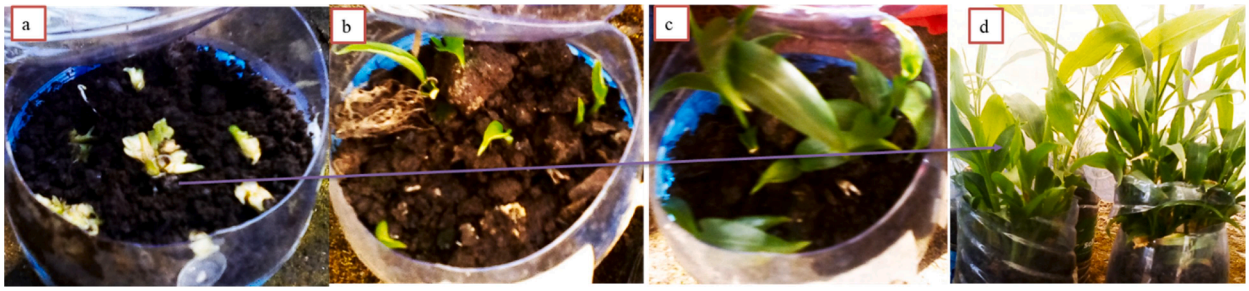

Fig. 4. In vivo performance of micro-rhizomes produced in vitro. (A) Micro-rhizome at planting, (B) sprouted micro-rhizomes after a month (C) sprouts on growth after 15 days of emergence, and (D) plants on vigorous growth after 2 months of emergence.

the work reported in this paper.

Data availability

Data will be made available on request.

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