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Establishment of a Simple Plant Regeneration System Using Callus from Apomictic and Sexual Seeds of Guinea Grass (*Panicum maximum*)

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Authors' contributions

This work was carried out in collaboration by the authors. Authors CL, ZJ and XC designed the study and author CL wrote the first draft of the manuscript. Authors NY and UK performed the experiments and data management. Authors ZJ and XC managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: For analysis of apomixis genes, as the first step, an efficient and simple plant regeneration system has been established using callus from apomictic and sexual seeds of guinea grass (*Panicum maximum*).
Study Design: The best basic medium for callus formation from matured seeds of guinea grass was selected, and then, the best combinations of growth regulators on different media were selected for plant regeneration by indirect organogenesis.
Methodology: Guinea grass accessions of sexual and apomict of seeds were used for culture. Seeds sterilized were cultured in Murashige and Skoog [10] medium (MS) and in that proposed by Chu et al. [12] (N6D) for callus formation. The best medium and, the effects of L-proline and growth

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regulators' type and concentrations on callus formation and plant regeneration in 3 accessions were examined, respectively. After the plantlets rooting in MS hormone-free medium, the complete plants were planted in pots for hardening.

Results: N6D medium has given better rates for callus formation, that is, 97.1% in sexual N68/96-8-o-5, and 91.7% and 84.6% in apomict N68/96-8-o-11 and 'Natsukaze', respectively. MS medium with 0.2 mg/l of Kinetin has given the best rate or plant regeneration among the used 4 kinds of the media. For each material, the best results were obtained on MS with 0.2 mg/l of Kinetin for N68/96-8-o-5, and MS with 2.0 mg/l of L-proline and 0.2 mg/l of Kinetin for 'Natsukaze'. After hardening of the regenerated plants in soil, 100% of surviving rates were obtained, and showed normal growth comparing with the plants-derived from seeds.

Conclusion: We have established, as the first case, an efficient and simple plant regeneration system by using callus from apomictic and sexual seeds of guinea grass for analysis of apomixis genes, consisting of analysis of the best media, L-proline usages and phytohormone combinations for callus formation, plant regeneration and hardening in different stages, respectively.

Keywords: Apomixis; apomixis specific gene; In vitro culture; culture medium; growth regulators; Panicum maximum; mature seeds.

1. INTRODUCTION

Guinea grass (*Panicum maximum*) is an important tropical forage crop. It represents in nature by both of tetraploid biotypes that can reproduce through apomixis, and sexual one [1]. Apomixis is a unique reproductive mode that by passes meiosis to produce seed genetically same to the mother parent [2]. So it is expected to promise the economic benefits over than the "Green Revolution" by using apomixis to fix F_1 plants as a variety in agriculture [3]. Up to now, we based on the results of cytological observation of embryo sac formation in both of sexual and apomictic guinea grass (*Panicum maximum*) by using Nomarski differential interference-contract microscopy (DIC) [4,5] and used differential screening method based on the ovary length as an index, and have isolated successfully the apomixis specific gene (*ASG-1*) [6] which specifically expressed in the stage of appearance of aposporous embryo sac initial cell (AIC) [7]. The application of apomixis as a technology to "clone" will be a good idea for plants that express genes homologous to the *ASG-1*, isolated from facultatively apomictic guinea grass [6], exerting the same function in similar tissues of embryo sac and surroundings. In order to do functional analysis of *ASG-1*, using gene transformation method mediated by *Agrobacterium*, the efficient and simple plant regeneration system must be established from the donor plant, that is, guinea grass. In addition, we have made the *ASG-1* transgenic plants in rice and *Arabidopsis* [8,9]. However, the systems from the most important and necessary plants of guinea grass have not been established in efficient plant regeneration and transformant using *Agrobacterium*. In this study, we focused on establishing system of plant regeneration from matured seeds of accessions of sexual and apomict and apomictic variety of guinea grass.

2. MATERIALS AND METHODS

2.1 Plant Materials

Guinea grass (*Panicum maximum* Jacq.) accessions of sexual N68/96-8-o-5, and apomict of N68/96-8-o-11 from which the *ASG-1* was isolated, and apomictic variety of "Natsukaze", cultured in Field Center of Minami Kyushu University, were used as materials for seed donor, respectively. The seeds were collected from living plants, dried for about one week in desiccator, and used for culture. This study was carried out in Faculty of Environmental and Horticultural Science, Minami Kyushu University, between April 2013 and October 2014. For seed sowing, at first, the matured seeds of 5g were put into the 50 ml tube containing 2.5% concentration of sodium hypochlorite solution, and 0.1% of tween 20, and with shaking in 120 rpm at room temperature for 3 hours for sterilization (Fig. 1A). And then, the tube was moved into biohazard and washed with sterilized water up to the bubbles faded away. The seeds were put onto the filter paper in the dish, and dried by using tweezer to take off the palea (Fig. 1B). As the preliminary examination, when the matured seeds just harvested were sowed immediately into the callus formation media, the germination of the seeds was not uniform. According to the phenomenon, if the seeds were used for gene transformation, it is worried that the transgenic plants would also be not uniform.

When we used the seeds having already been harvested for over 3 months kept in laboratory room, the uniform germination was obtained from the seeds. From this result, it is considered that the seeds of guinea grass need breaking of dormancy, so that we used the seeds over 3 months keeping in room temperature for giving uniform germination.

2.2 Culture Media for Callus Formation

The seeds were put into the dishes, respectively containing the Murashige and Skoog (MS) medium [10] with 30,000 mg/l of sucrose, 5 mg/l of 2,4-D, 3,200 mg/l Gellan Gum, pH 5.8 [11] and Chu et al. [12] (N6D) medium with 30,000 mg/l of sucrose, 5 mg/l of 2,4-D, 3,200 mg/l Gellan Gum, pH 5.8 [11], and N6D medium [12] with 30,000 mg/l of sucrose, 3,981 mg/l of CHU powder [12], 300 mg/l of casamino acid, 2,878 mg/l of L-proline, N6D vitamins, 2 mg/l of 2,4-D, 8,000 mg/l of agar, pH5.8. The culture was kept in 30°C, dark for callus formation in interval subculture of 3 weeks.

After one month of culture of seeds, the rates of callus formation and the difference in the rates among sexual of N68/96-8-o-5, and apomicts of N68/96-8-o-11 and 'Natsukaze' were examined on the N6D medium. For each accession, 100 seeds were used for culture and the same experiment was repeated for three times.

2.3 Culture Media for Plant Regeneration

In order to find out the best medium for plant regeneration, the calli derived from matured seeds of N68/96-8-o-11 were cultured on different regeneration media. As there were not proper medium reported previously for guinea grass, the examination of the efficient condition was carried out on four kinds of media [13,11,14,15] (Table 1), which were used for plant regeneration from different tissues (not seeds) of monocotyledon plants.

For the calli of N68/96-8-o-5, N68/96-8-o-11 and 'Natsukaze', MS medium with 30g/l of sucrose, 0.2 mg/l Kinetin, pH5.8, and 8 g/l agar was used to examine the regeneration rates. In addition, an essential amino acid of L-proline (2 mg/l) was added into MS medium and used for the 3 same materials. As in the pre-treatment, different effects were observed in the 3 kinds of materials, the best combination of growth regulator types was also examined. The regeneration rates were investigated in MS medium with 2 mg/l of Kinetin, and 0.1, 0.2 and 0.3 mg/l of naphthalene acetic acid (NAA), respectively. For each accession, 30 calli were cultured and the same experiment was repeated for three times.

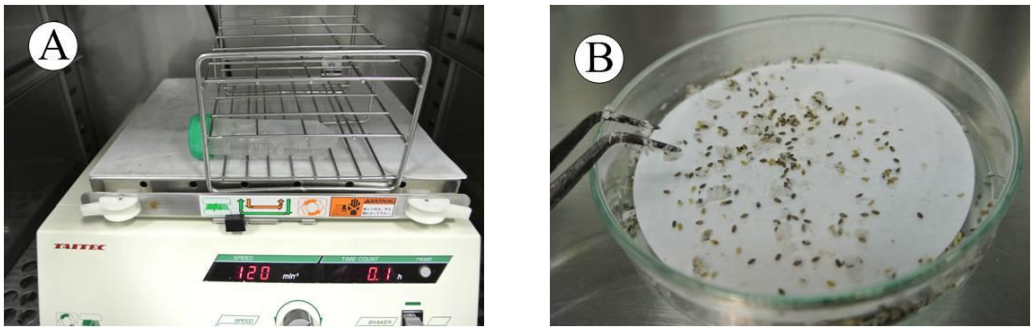

Fig. 1. The matured seeds of guinea grass treated for germination. A: seed sterilization by shaking machine; B: seeds in the dish after washing by sterile water

Table 1. Comparison of the best combination of growth regulators reported previously in different materials used in this study for plant regeneration from callus derived from matured seeds of guinea grass*

Kinds of materials	NAA	GA_3	Kinetin	BAP
Rice [13]	0.02 mg/l	-	2.0 mg/l	-
Guinea grass [11]	0.01 mg/l	-	2.0 mg/l	-
Switch grass [14]	0.2 mg/l	0.5 mg/l	-	1.0 mg/l
Toll fescue [15]	-	-	0.2 mg/l	-

**Murashige & Skoog [10] medium was used as the basic medium*

2.4 Hardening of Regenerated Plantlets

After the plantlets were cultured in MS with growth regulators free medium for 2-3 weeks, the complete plants with well-developed roots were planted in pot containing metro mix (Hyponex, Japan) and vermiculite 1:1 (v:v) for hardening under the conditions of 30°C, 24 hours of day light. The same experiment was repeated for three times.

3. RESULTS AND DISCUSSION

3.1 Evaluation of the Culture Media for Callus Formation from Matured Seeds

For the examination of media on callus formation in guinea grass, Chen et al. [11] have used the basic MS medium complemented with 5~10 mg/l of 2, 4-D for the leaflet culture. In this study, the same medium gave the slow growth of callus compared with that of N6D, even though the callus formed as same in both media (Fig. 2A, B). In the N6D which gave higher rates of callus formation, L-proline was added with 2.878 mg/l. Li & Qu [14] also indicated that the addition of L-proline is efficient to callus formation used for the transformation of switch grass. Therefore, the addition of L-proline and N6D medium which is well used in rice culture were adopted in this study for callus formation from matured seeds of guinea grass.

3.2 Difference in Callus Formation Rates between Sexual and Apomict

Chen et al. [11] has reported that there have differences between the accessions and between sexual and apomict in cultures of leaflets of guinea grass. In the culture of matured seeds there also appeared differently in callus formation (Figs. 3A, B, C). In sexual N68/96-8-o-5 accession, while the seed germinated designated as in Fig. 3D, the callus located in the root pole, derived from embryo formed, and later, the callus showed vigorous growth in sizes of the big callus and small young leaflet. However, in apomicts of N68/96-8-o-11 and 'Natsukaze', while the seeds germinated designated as in Figs. 3B and C, the shoots grew firstly and fast, and then, callus formed in the root pole, after the transition from shoot to callus. And as a contrast to the sexual, the callus of apomict showed the sizes of small callus and big young leaflet. The difference of callus formation appeared between sexual and apomict may be considered as the difference of growth regulators' balance existed in both of them.

In the culture of matured seeds there also appeared differently in callus formation (Fig. 3A, B, C). In sexual N68/96-8-o-5 accession, while the seed germinated the callus derived from embryo formed, and later, the callus showed vigorous growth. However, in apomicts of N68/96-8-o-11 and 'Natsukaze', the shoot grew firstly, and then, callus formed from around root and young leaflet. The calli formed in all the 3 accessions showed white color and soft characteristics, excepting the apomict 'Natsukaze' which showed compact one in part of calli (Data not showed). However, all the 3 accessions gave high rates of callus formation after one month of culture. In particular, the sexual N68/96-8-o-5 gave the highest rate of 97.1% (Table 2), comparing with those of 91.7% and 84.6% in apomicts of N68/96-8-o-11 and 'Natsukaze', respectively. In this study, the fact that the sexual one showed vigorous growth of callus (Fig. 3D) and highest rate, can be considered as the important and positive factor to the further transformation experiment of *ASG-1* for its functional analysis, even though the reasons were not yet clear.

Table 2. The rates of callus formation from matured seeds in N6D medium

Accessions	Rates of callus formed (%)
N68/96-8-o-5	97.1±9.2(165/170)[z]
N68/96-8-o-11	91.7±12.5(266/290)
'Natsukaze'	84.6±11.9(237/280)

[z] *(Number of callus formed/number of seeds cultured)*

3.3 Examination of Callus Culture Media for Plant Regeneration

Fig. 3 shows callus formation in different accessions in the same medium, and showed the difference in callus formation after one and two months of culture. Fig. 3D showed calli after two months of culture in which, seedlings were disappeared gradually. However, when the calli were moved onto medium for plant regeneration, only calli without seedlings were used (Fig. 4).

Table 3 showed the rates of plant regeneration in different media from calli-derived from matured seeds of guinea grass. There appeared with brown color in callus cultured in the Rice medium [13], a well-used one in rice, and no color change and no growth in callus in the medium used in

switch grass[14]. And more, there appeared with only root formation in RE medium [11] used in guinea grass. However, the shoot was regenerated in the medium used in tall fescue [15]. Therefore, it is considered that MS complemented with 0.2 mg/l of kinetin is the best selection for plant regeneration from the calli-derived from matured seeds of guinea grass (Figs. 4A, B, C and D).

3.4 The Effects of L-proline and, Growth Regulator Type and Concentration on Regeneration Rates of 3 Kinds of Guinea Grass

Table 3 shows the results of effects of growth regulators and L-proline on the rates of plant regeneration. When L-proline was not used in the same MS medium, the lowest rate of plant regeneration was 25.0% in apomict of N68/96-8-o-11, and the highest one was 80.0% in sexual N68/96-8-o-5. And the same apomict of 'Natsukaze' gave the rate of 61.9% higher than that of N68/96-8-o-11. From those results, it is clear that the rates of plant regeneration are different among the accessions and variety used in this study.

To make it clear further that how L-proline effects the regeneration rate, 2.0 mg/l of L-proline (Table 3) was added for regeneration on MS medium. As a result, N68/96-8-o-5 showed no any regeneration with shoot and root, even though it gave the highest regeneration rate of 80.0% among the 3 accessions when the medium was added only with 0.2 mg/l of kinetin. However, the apomict of N68/96-8-o-11 gave the rate of 41.6% higher than the 25.0%, when the L-proline was not added. And more, the apomictic variety of

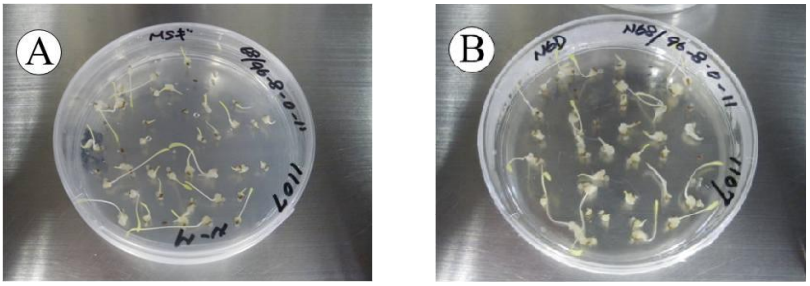

Fig. 2. Callus formation from the matured seeds of guinea grass cultured in different media. A: callus formed in MS medium; B: callus formed in N6D medium

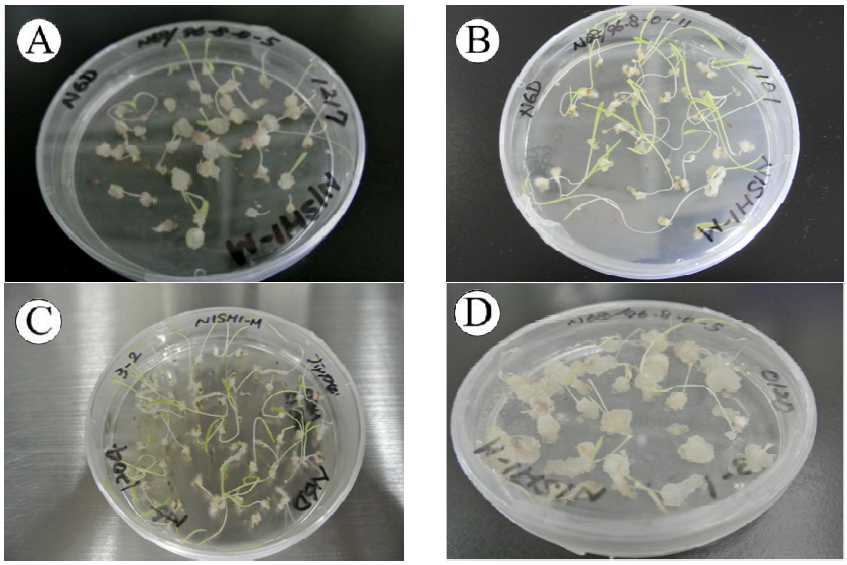

Fig. 3. Morphologies of calli formed in 3 accessions of guinea grass. A: calli in N68/96-8-o-5 after one month culture; B: calli in N68/96-8-o-11 after one month culture; C: calli in 'Natsukaze' after one month culture; D: calli in N68/96-8-o-5 after two months of culture

'Natsukaze' gave the highest rate of 91.7%. From the results obtained, L-proline may play a positive role for plant regeneration in apomicts but sexual one, even though the relation between L-proline and apomict was not clear. The materials of sexual and apomict used in this study were few, so that the further experiments are needed for understanding the effect of L-proline on the plant regeneration in apomicts.

As the above different results obtained from the different accessions, the best combinations of growth regulators including L-proline and NAA (Table 3) on MS medium for each accession was further investigated. Fig. 5 shows the plant regeneration from different combinations of growth regulators in different accession. There were not different morphologies observed among the different media. And from Table 3, it is clear for the best combinations of growth regulators on MS medium for each accession that, the best results of 80% in N68/96-8-o-5, 91.7% in 'Natsukaze', and 41.6% in N68/96-8-o-11 were obtained in 1) Kin:0.2 mg/l, 2)L-proline:2.0g/l and Kin:0.2 mg/l, and 3)L-proline:2.0 g/l, NAA:0.2 mg/l, and NAA:0.3 mg/l and Kin:0.2 mg/l, respectively. These results indicated that there existed in different regeneration rates among different materials and different combinations of growth regulators even on the same medium.

3.5 Hardening of Regenerated Guinea Grass

In general, a lot of regenerated plantlets need to be moved onto growth regulator free medium for rooting in direct or indirect organogenesis types, and then, for hardening. In this study, as the plantlets without rooting or with weak and few roots were obtained from callus derived from the matured seeds, it is clear that the type of plant regeneration should be indirect organogenesis. As described in M & M, the plantlets obtained were moved onto growth regulator free MS medium, and the whole plants with rooting were obtained in rates of 100% (Fig. 6A). As guinea grass is weak to high humidity condition, the regenerated plants with shoot and roots were transplanted into the 6 cm diameter of pot containing metro mix (Hyponex, Japan) and vermiculite (in 1:1 = v : v) for hardening under 30°C, 24 hours of day-length in a growth chamber. All of the plants obtained from all of media were subjected to acclimatization. As a result, all of the treated plants gave 100% of surviving rates, and showed normal growth comparing with the plants-derived from seeds (Fig. 6).

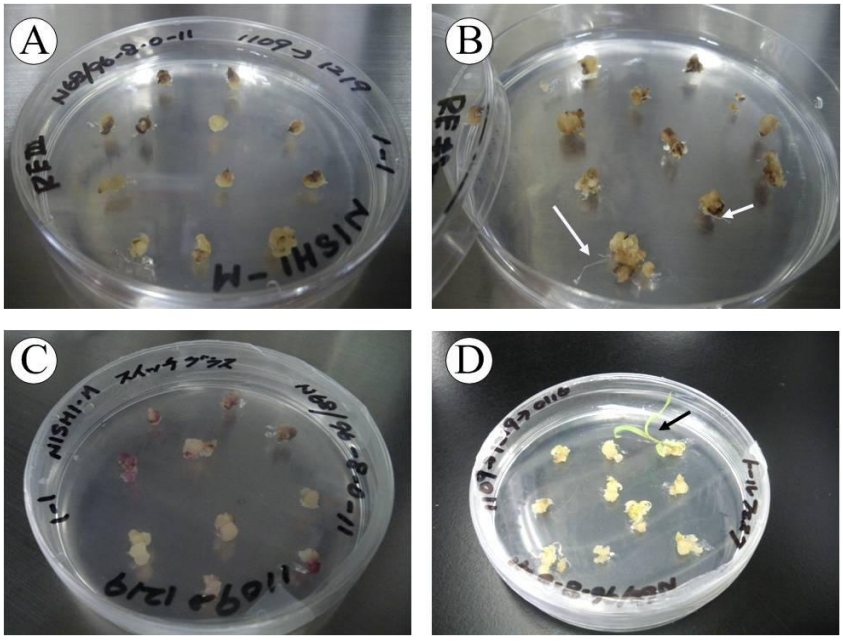

Fig. 4. Plant regeneration on 4 kinds of phytohormone combinations in MS media in different accessions. A: medium for rice[13]; B: medium for guinea grass[11]; C: medium for switch grass[14]; D: medium for toll fescue[15], referring to Table 1

Table 3. The effects of L-proline and NAA on the rates (%) of plant regeneration of guinea grass[z]

Accessions	Kin:0.2 mg/l	L-proline:2.0 g/l Kin:0.2 mg/l	NAA:0.1 mg/l Kin:0.2 mg/l	NAA:0.2 mg/l Kin:0.2 mg/l	NAA:0.3 mg/l Kin:0.2 mg/l
N68/96-8-o-5	80±10.4	0±0	8.3±3.4	16.6±3.6	0±0
N 68/96-8-o-11	25±6.4	41.6±5.7	8.3±2.5	41.6±8.8	41.6±7.9
'Natsukaze'	61.9±5.8	91.7±9.2	0±0	25.9±5.7	48.3±6.7

[z]*Murashige & Skoog [10] medium was used as the basic medium; For each accession, 50 calli were cultured, respectively and the same was repeated 3 times*

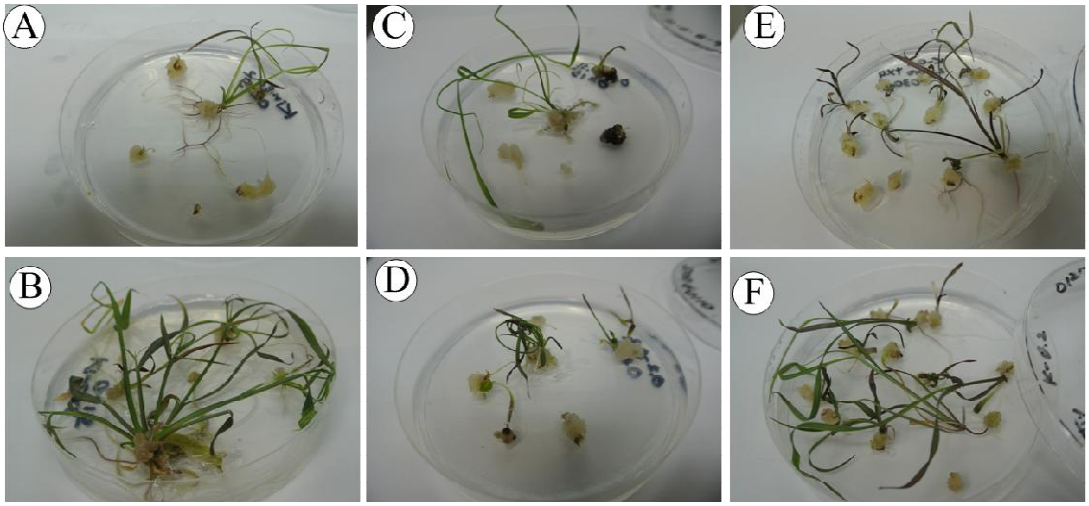

Fig. 5. Plant regeneration of guinea grass in different accessions and media. A & B: N68/96-8-0-5 (MS+0.2 mg/l : Kinetin); C & D: N68/96-8-0-11 (MS+0.2mg/l : NAA, 0.2 mg/l : Kinetin); E: 'Natsukaze' (MS+0.2 mg/l : Kinetin); F: 'Natsukaze' (MS+2.0g/l : L-proline+0.2mg/l : Kinetin)

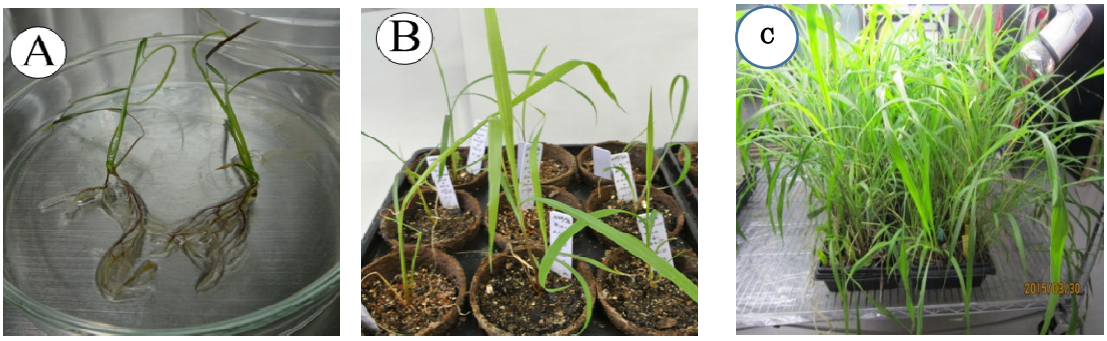

Fig. 6. Hardening of regenerated plants of guinea grass. A: The regenerated plants before hardening; B: The plants after hardening; C: The whole plants are successfully hardened

4. CONCLUSION

We have established an efficient and simple plant regeneration system by using callus from apomictic and sexual seeds of guinea grass for analysis of apomixis genes, consisting of analysis of the best media, L-proline usages and growth regulator combinations for callus formation, plant regeneration and hardening in different stages, respectively. We are starting to do the *ASG-1* transformation with the established plant regeneration system.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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