Tissue Culture Propagation of "Hausa potato" (*Solenostemon rotundifolius* (Poir) JK Morton)

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Abstract

We have developed an effective plant propagation method of 'hausa potato' through shoot proliferation *in vitro*, acclimatization in the greenhouse and transplantation of the plantlet on the individual media container for planting material production. The shoot cultures were initiated from shoots that grew from tuber collected from the field, disinfested using standard method and transferred to MS medium with 1 mg/l of BA for shoot proliferation. The proliferating shoots were then subcultured to MS medium with no growth regulators to induce rooting and enlarge the shoot. The plantlets were acclimatized in a plastic box containing medium of a mixture of cocopeat, sands, rice husk charcoal and top soil covered with plastic film to maintain humidity. The plantlets survived at 85% and almost 100% grew into planting materials ready for transplanting in the soil. The planting materials survived in the field and produced tuber normally.

Key words: *Solanostemon rotundifolius*, hausa potato, kentang hitam, *in vitro* propagation, acclimatization, planting materials.

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Introduction

'Kentang hitam' with vernacular name 'hausa potato' (*Solanostemon rotundifolius (Poir) JK Morton)* is not potato but a member of the *Lamiaceae* family that produce small tuber that contains alternative source of carbohydrates. It is native to tropical Africa but cultivated in various regions in Asia including India, Pakistan and Indonesia (Jansen, 1996). Hausa potato is a perennial herbaceous and grown in Java, Bali and Madura (Heyne, 1987). The tuber of hausa potato is mostly to be found in traditional markets on harvest seasons of June to August. The tuber is available but not abundantly and sold for around Rp 2,500 to Rp 6,000 rupiahs per liter, which is slightly more expensive than most tubers like sweet potatoes and cassavas. These tubers are consumed as vegetables (Nugraheni & Astuti, 2011), boiled or as a mix course and serves as a substitute for potatoes most people usually consume. Starches from the tubers of hausa potato could be used as an additional component for pharmacy.

Basically, these potatoes are cultivated by cuttings or tubers that remain in the ground. Usually, planting materials are scarce because they are not specifically propagated. The use of cuttings without a thorough inspection of the tubers could also affect the quality of the planting material. The alternative utilization of cuttings through the techniques of tissue culture could be tested for producing high quality planting materials.

Multiplications through *in vitro* by induction and the multiplication of lateral buds have some advantages, i.e. efficiency of time and lands. Additionally, this technique is required for the multiplications of genotypes developed through both genetic manipulations and genetic engineering. Manipulations of somatic cells by induced mutation techniques followed by *in vitro* selection, protoplast fusing or even genetic engineering via plant regeneration through organogenesis could be utilized for assisting plant breeding to increase the genetic variability.

The multiplication *in vitro* of species from the family of *Lamiaceae*, similar to hausa potato, has been reported and included species from the genus of *Mentha* (Rech & Pires, 1986), *Mentha piperita* (Sunandakumari *et al*., 2004; Vasile *et al.,* 2011). Shoot proliferation

and organogenesis of hausa potato (Hoesen, 1991) and *in vitro* conservation (Novianti *et al*., 2003) had been reported, but not their survivals in the field.

We report here a workable protocol for micropropagation of hausa potato from initiation of the culture to acclimatization and the growth of planting matrerial in the nursery.

Materials and Methods

Growth medium and growth environment of the culture. The basal medium used was MS formulation (Murashige & Skoog, 1962) which was modified to contain (in mg/l): NH_4NO_3 1,650, KNO_3 1900, $CaCl_2.2H_2O$ 440, $MgSO_4.7H_2O$ 370, KH_2PO_4 170, minor salts, vitamine (Glycine 4.0, Pyrodixine 1.0, Thiamin 0.2, Nicotinic acid 0.5), sugar 30,000, and growth regulator BA according to the treatments. Medium was gelled with Gelrite 3.0 g/l of gellan gum. The pH of the media were set to 5.7-5.8 with 0.1 N of HCl or KOH prior to addition of the gelling agent. The gelling agent in the media were dissolved by heating inside a microwave oven and the distributed in a 25 ml aliquots into a 200 ml glass vessels, covered with two layers of plastic film and secured with a rubber band. The media were then aotoclaved at 121^{o}C, 15 psi for 20 min. The growth media were then stored in a cupboard in a room of 25^{o}C for at least a night before use. The cultures were kept at a culture room of 27^{o}C, on a rack with light intensity of 261-352 lux for 16 hours photoperiode.

Culture Initiation. Tubers harvested from the field of three accessions of 'Bogor 1', 'Solo 1' and 'Nganjuk 1' were washed and puton trays layered with wet towel papers and kept in a room. The tuber were kept humid by spraying with tap water as needed. After 2-3 weeks, buds and small shoots grew from the tubers and they are used as explants (Figure 1A). Shoots of 1-2 cm length were excised from the tubers with a piece of tuber attached to the shoot were washed with running tap water until visually clean. The explants were soaked in 50% teepol for10 min and rinsed with tap water. Explant were then soaked in outoclaved distilled water and brought to laminar air flow cabinet for disinfestation procedure. Explants were then soaked in solution of bleach (Bayclin, NaOCl 5.25%) of increasing concentration of 20% for 10 min, 10% for 10 min, 5% for 15 min in which after each soaking the explants were rinsed thrice with autoclaved distilled water. The explants were then excised to make a 1-cm long nodal explants with leaf trimmed off. The nodal explant were inoculated to MS medium with 0.5 mg/l of BA. These initiation experiments were carried out four times for accession 'Nganjuk 1'. The initiated cultures were observed at the 1st and 2nd week for contamination and growth. The uncontaminated cultures of 4-6 week old were then subcultured at the same medium for several passages to reach certain number, then subcultured to medium without growth regulator to reach enough number for further experiments.

The effect of BA concentrations on shoot proliferation. Shoot proliferation is studied on MS basal medium similar to initiation medium but with treatment of concentration of BA (0; 0.1; 0.5; 1.0; dan 5.0 mg/l). Inoculum to be used in this experiment was nodal explant of 1 cm from 6-8 week old shoot culture of accession Bogor 1, Solo 1 and Nganjuk 1. The basal of the inoculum was inserted about 5 mm deep in the medium with erect position. Each treatment consisted of five vessels as replicates and each vessel contained three inocula. The growth of the culture were observed at the end of week 4 and week 7 after inoculation on survival percentage, number and length of shoots, number of nodes and rooting percetage. Data were presented as histogram of average and standard error to differentiate among treatment.

Acclimatization of plantlets and plant growth in the nursery. Plantlets of 6-8 week old grew on MS without growth regulators and had a lot of roots and vigorous were removed from the vessels by adding water to agar to minimize the root damage. The plantlets were then rinsed with running tap water to further removed any agar that adhere to the roots. The planlets were soaked in 1.0 g/l of fungicide Benstar (with active ingredient of benomyl) and 1.0 g/l of Agrept (agrymicyn), each for 3 min. The planlets were then planted in rectangular plastic basin of 40×30 cmfilled to 1/3 deep with 6-h steamed acclimatization medium of different composition: a) cocopeat :

sand : rice husk : top soil =9:5:2:1, b) cocopeat : sand : rice husk = 5:5:3, c) cocopeat : sand : rice husk : top soil = 2:2:2:1. The planlets were sprayed with 2 g/l of fertilizer solution Growmore (N-P-K=15-15-15), and the basin were covered with transparant plastic film and secured with rubber strips prepared from bike inner tubes. The plantlets were maintained in a greenhouse with averagedtemperature of 29°C, and light intensity of 600-2,600 lux. The plantlets were maintained by spraying with water when needed and kept for over 4 weeks until new shoots grew. The number of plants measured or counted for observation were 20 for each basin, and there were 2 basins per treatment. The variable to be observed include survival, cause of death, height increament, number of leaves. The data were presented as mean and standard error and presented as graphs. Plantlet post acclimatization of 4-5 weeks were transplanted to polibag of 10×20 cm filled with mixture of soil: compost: rice husk coal = 4:2:5:1. The planlets in the polibag were covered with shading net of 50% and watered when needed. The plantlets were maintained in a greenhouse with averaged temperature of 30.2°Cand light intensity of 230-4,510 lux. The variable to be observed include survival, cause of death, height increament, number of leaves. The number of plants measured or counted for observation were 33 for each treatment. The data were presented as mean and standard error and presented as graphs. After 1 month, the plantlets had grown to young plants and ready for transfer to the field.

Results

Hausa potato can be easily mass propagated via *in vitro* technology and the resulting planting materials could be planted in the field dan produced tuber normally. The protocols can be described as follows. Culture initiation was done using shoots that grow from tuber (Figure 1A). The resulting shoot cultures is characterized by the growth of multiple shoots with some roots and healthy and prolific leaves (Figure 1B). The separation of shoots some of which with roots resulted in planlets without the need of a separate rooting stage (Figure 1C). Plantlet are acclimatized in plastic basins containing a mixture of media covered with plastic film which was removed about 1-2 weeks before transplanting (Figure 1D). The post acclimatized planlets grow well in polibag containing medium (Figure 1E) that survive planting in the field (Figure 1F) and producing tuber (Figure 1G).

Culture initiation *in vitro*

After three days of culture, contamination of fungi or bacteria began to appear in 'Nganjuk 1" accession. Contamination could appear even after the 2nd week of culture. Contamination from four independent initiation trials ranged 14-61% (Tabel 1). Some of the cultures turned brown and then died or remained green from which grew new shoots ranged from 6-57% (Tabel 1). Percentage of contamination and explant growing shoots varied greatly among trial and indicating no trend.

The effect of BA concentration on shoot proliferation of "hausa potato *in vitro* at 4 and 7 weeks after culture

The effect of concentration of BA on the growth of shoot culture was noticeable at age 4 weeks and become even more significant at age 7 weeks (Figures 2 and 3). The growth of the shoots were effected by the concentration of BA in the medium. In the medium with no BA, the shoot cultures were characterized by the growth of leaf to extensive size with vigorous stem and branching, while at higher concentration of BA, the height of the shoot decreased but the shoots clustered indicating highwer number of shoots (Figure 2).

Quantitatively, the respone of number of buds tend to be parabolic to BA conccentration which reached maximum at 1.0 mg/l and then decreased at 5.0 mg/l. This parabolic response occurred on all the three accession tested at week 4 (Figure 3 A, left) and remained unchanged at week 7 (Figure 3 A, right) for the accession 'Bogor 1', the decrease of number of shootscould not be demonstrated due to missing data of contamination. The age of culture tends to increase the maximum number of shoots for 'Bogor 1' and 'Nganjuk 1' but not for 'Solo 1' (Figure 3 A, left and right).

The length of shoot decreased with increasing concentration of BA in the medium for all accessions tested and this trend remained stable at age 4 and 7 weeks (Figure 3 B, left and right). The trend of decreasing shoot length varied with accession, for

example, at 4 weeks after culture, the shoot length decreased significantly at BA of 0.1 mg/l for 'Bogor 1', but for 'Solo 1' and 'Nganjuk 1' significant decline occurred at 5.0 mg/l BA.

Tabel 1. The fate of Nganjuk 1 explant two weeks after initiation.

Initiation no.	Contaminated explant (%)	Browning explants (%)	Explant with shoots (%)	Kind of contamination
1.	14.28	28.57	57.14	Bacteria
2.	61.11	0.00	38.89	Bacteria and fungi
3.	15.15	78.79	6.06	Bacteria and fungi
4.	33.33	60.00	6.67	Fungi

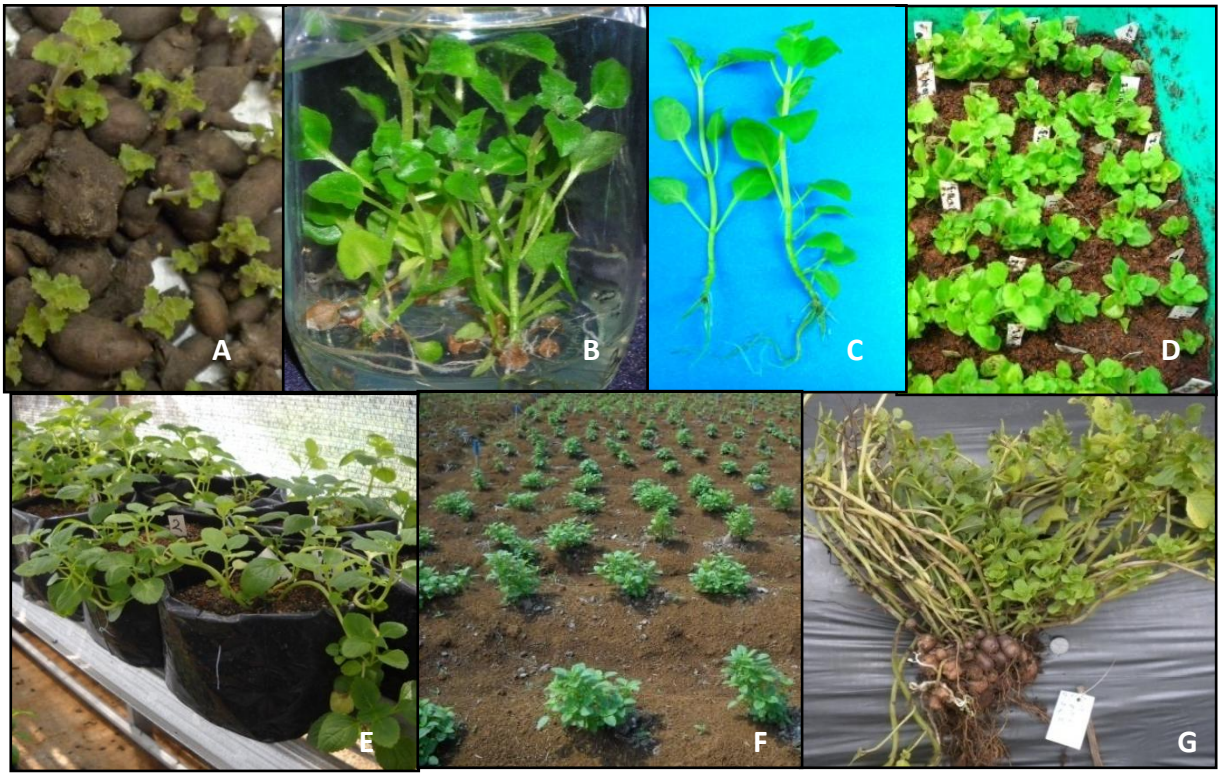

Figure 1. Propagation of 'hausa potato' through tissue culture. A) shoot that grew on the tuber was used as explants, B) shoot cultures, C) planlets ready for acclimatization, D) planlets had grown during acclimatization, E) planting materials in polibag containing medium, F) tissue culture-derived plants in the field, G) the plant and the tuber it formed in the field.

Figure 2. The effect of BA concentration (from left to right: 0, 0.1, 0.5, 1.0 dan 5.0 mg/l) on the shoot proliferation *in vitro* of hausa potato of accession 'Nganjuk 1'.

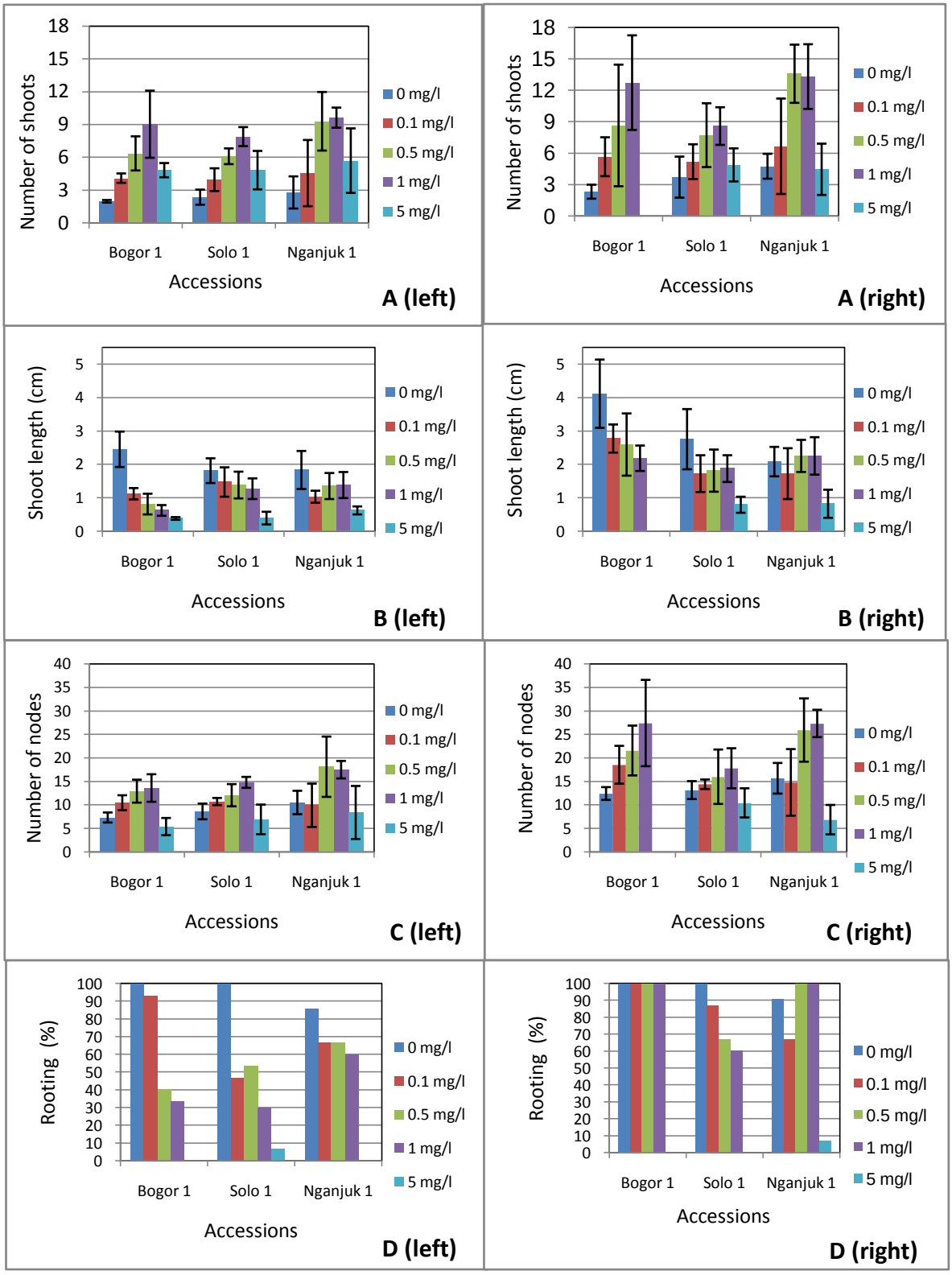

Figure 3. The effect of BA concentrations on the proliferation of three accessions of 'hausa potato' *in vitro* at 4 week (left) and 7 week (right). (A) Number of shoots, (B) shoot length (cm), (C) number of node, and (D) rooting percentage.

Parabolic response against BA concentration was also observed for variable of number of node at 4 weeks (Figure 3 C left) and the whole response shifted up at 7 weeks (Figure 3 C right). This response is similar to that ofnumber of shoot response.

The increasing concentration of BA decreased number of root that formed at age of

4 weeks, and at the concentration of 5.0 mg/l of BA tend to inhibit the root development almost completely (Figure 3 D left) even after three weeks later (Figure 3 D right). The inhibition of root formation with the increasing BA concentration varied with accession. For example, 'Nganjuk 1 showeddegrees of root grow inhibition by BA at age 4 but at age 7 weeks, the percent root formation reached 100% for concentration of BA to 1.0 mg/l, but for 'Solo 1' the percentage of root formation remain gradually decreasing, while for the 'Bogor 1', the response was somewhat irregular at week 7 (Figure 3 D right).

Acclimatization and growth of the plants in the nursery

Percentage of survival of the acclimatized shoots tend to decrease with time but not significantly eventhough there were indication that medium 1 resulted in more stable survival rate compared to other medium. The lowest survival rate averaged over 80% in medium 1, and was 70% in medium 2, but only about 50% in medium 3.

Basal rot of the planlet is mostly the cause of death. The plant height was not significantly diffferent among medium treatment, but increase linearly with time (Figure 4B) and reached the height of 15-20 cm at week 4. The responds of number of leaves was similar to the plant height, that was linear with time and not significantly different among medium treatment (Figure 4C). Visually the plant grown in medium 1 showed better performance compare to other medium, but this medium dried fast.

Upon transfer to individual media-containing polybag, the acclimatized plantlets of accession 'Solo 1' survived at nearly 100% (Figure 5A) and grow linearly with time in regard to plant height (Figure 5B) and number of leaves (Figure 5C) in the nursery in the greenhouse and therefore had grown into planting materials. The planting materials from the nursery were succesfully established in the field and reach maturity and produce tubers in 4-6 months (Figure 1 F, G).

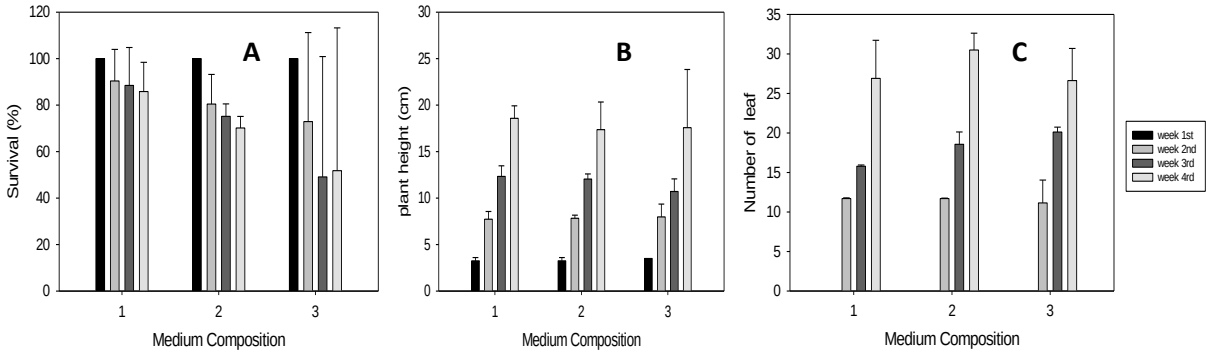

Figure 4. The growth of planlets during 4 weeks acclimatization of accession Solo1 in three different medium compositions. Medium composition were cocopeat: sands: rice husk charcoal: top soil with ratios of 1) 9:5:2:1, 2) 5:5:3, 3) 2:2:2:1. (A) percent of survival, (B) plant height (cm), and (C) Number of leaves.

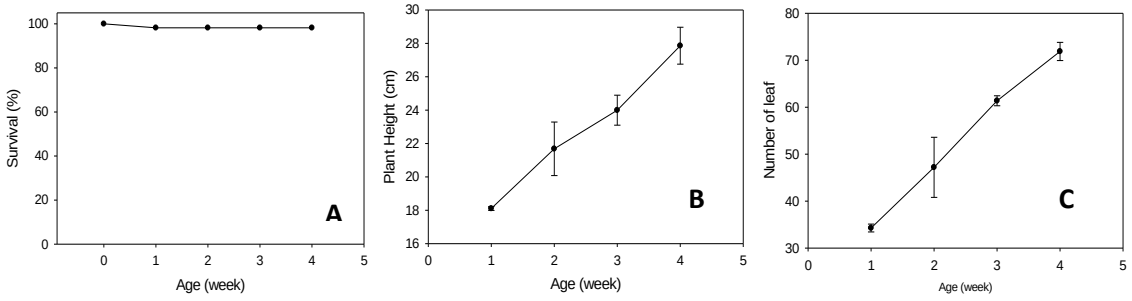

Figure 5. The growth of planting material in individual containers of accession ' Solo 1' for 4 weeks.(A) percent of survival, (B) plant height (cm), and (C) Number of leaves.

Discussion

The culture could be succesfully initiated using a standard disinfestation method involving the use of commercial bleach with gradually decrease of concentration, eventhogh bacteria and fungi contamination rate were high and so did the culture death. The soft and herbaceous tissue may have not withstand the high end of standard concentration of 20% bleach (George, 1993).

The shoot proliferation of the hausa potato was significantly affected by the concentration of BA in the medium, and that the responds were parabolic indicating that the concentration tested covered suboptimal, optimal and supraoptimal concentration (Witjaksono, 1985; Geroge, 1993). BA is a synthetic growth regulator acting similar to kinetin which has high activity and inexpensive and, therefore, commonly used for micropropagation of wide arrays of plants and this can be easily seen from extensive tabulated published reports for example in the book by George (1993). The optimum concentration of BA at 1.0 mg/l for shoot proliferation is also common for a lot more other species. This experiment is also similar to the results of Saha *et al.* (2010) in which the optimum BA concentration for multiplication of*Ocimum kilimandscharicum* was 1.0 mg/l with maximum number of shoot per explant of 6.09. Similarly, Echeverrigaray *et al.* (2010) reported that number of shoots of *Salvia guaranitica* Benth. increased to 4.4 per explant at 2.22 μM (0.5 mg/l) but decreased at 4.44 μM BA. Number of shoot also increased to 3.77 per explant on micropropagation of *Cunila insica* at 4.4 μmol/l BA, but decreased at 8.8 dan 17.6 μmol/l BA (Agostini & Echeverrigaray, 2006). For tissue culture of *Pogostemon cablin* Benth., number of shoots increase to 45.66 per exsplant at 0.5 mg/l BA but decrease at 1.0 mg/l BA (Swamy *et al.*, 2010). Similar parabolic response of shoot proliferation against concentration of cytokinin was also found in *Brassica oleracea* (Witjaksono, 1985). For the 'hausa potato' however, the growth of the shoot demonstrated multiple shoots even with the absent of plant growth regulator. This is in line with the plant habitus that branch at the base of the main stem close to soil.

With increasing number of shoot, the height of the shoot decrease with concentration of BA in the medium. This is also a common phenomenon as it is found for example in cauliflower, *B. oleracea* Botrytis Group (Witjaksono, 1985). Noviati *et al.* (2003) found that shoot length of hausa potato *in vitro* decreased from 2.6 cm to 0.65 cm as concentration of BA in the medium increase from 0 to 4 mg/l. Shoot culture of *Mentha piperita* L. enriched with BA at concentration of 0.5-2.0 mg/l showed a shoot length decrease from 7.4 cm to 5.5 cm (Vasile *et al.*, 2011).

The inhibition of root formation with increasing concentration of BA in the medium of 'hausa potato' illustrated the effect of sitokinin in counteracting the effect of auxin in the formation of root as pointed out by Skoog & Miller (1957) and later confirmed by a lot other publication (George, 1993).

The success of the acclimatization of the plantlet of 'hausa potato' could be attributed to microclimate of the box and the greenhouse in which the experiment were conducted.The temperature of 29°C and light intensity of 600-2,600 lux seems to be sufficient. The media that were consisted ofcocopeat (fiber of coconut husk), sand, rice husk coal with or without top soil may also sufficient in keeping the microclimate humid, and hold sufficient nutrient for root to absorb. These medium components were from locally available materials and inexpensive but serve all the purposes of nursery medium which were holding water (cocopeat), aeration (sands), holding nutrients (rice husk charcoal and soil).

The closure of the acclimatization container with plastic film allows sufficent humidity for the plantlet not to dry out and sufficient light for photosynthesis. In this stage, the plantlets are kept at humidity, light intensity and temperature similar to tissue culture condition, therefore the plantlets are only forced to photosynthesize. After a month, the cover was removed to force the plantlet to adapt to lower humidity. The tranplantation of the planlets in individual container was to provide for more root growth so that the plant could adapt better againsthumidity fluctuation.

This workable and complete micropropagation protocol of hausa potato from initiation of culture to production of planting materials that is proven to produce normally in the field is needed as the foundation for other manipulation *in vitro* or

genetic engineering for plant improvement (Leksonowati & Witjaksono, 2011; Witjaksono & Leksonowati, 2012). With these approaches, hopefully productivities ofunderexploited plants producing non-seed carbohydrates can be improved so that they attractfarmers to farm them that in turn improve food diversification and food security.

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