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Establishment of a One-Step Plant Regeneration System in Sweet Potato (*Ipomoea batatas* [L.] Lam.)

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Abstract

We investigated the efficiency of plant regeneration using diverse plant tissues on culture medium containing a combination of plant regulators in sweet potato. Ginhongmi and Sinwhangmi showed the highest callus induction rate among five cultivars of sweet potato tested. The highest shoot and root formation frequencies were acquired from calli derived from internode tissue in the MS medium with 1.0 mg/L NAA and 5.0 mg/L BA. The optimum condition of regeneration media depending on types of tissues was somewhat different, for example, the combination of 0.1 mg/L NAA and 2.0 mg/L BA produced the highest regeneration frequencies for the explants of young leaves. The highest induction of callus, roots and plant regeneration was observed on MS medium with NAA and BA. Taken together, this one-step plant regeneration system skipping hormone shift step can be exploited for the new plant breeding scheme of sweet potato.

Introduction

Sweet potato (*Ipomoea batatas* [L.] Lam.), which is a member of the family Convolvulaceae, genus Ipomoea, section batatas, is an important food crop that provides carbohydrates and protein to a large sector of the global population. Sweet potato is cultivated in tropical and sub-tropical areas of the world for its edible tuberous roots, which are high in starch and vitamins (Woolfe 1991). Young leaves and vine tips are also eaten, and are an excellent source of vitamins A, C, and B2 (Hill et al. 1992). Sweet potato also has the potential for use as a biomass species for methane and ethanol production and has therefore gained interest as a possible alternative to fossil fuels (Choi et al. 2008; Chu et al. 2012; Oyeleke et al. 2012; Ferrari et al. 2013). Sweet potato is hexaploid (6x=90), which makes it difficult to form seeds. Additionally, it is difficult to cultivate sweet potato under the temperate climate conditions of South Korea. Accordingly, it is necessary to secure a disease free stock and to overcome the limitations of traditional breeding. However, previous investigations conducted to enable plant regeneration and transformation of sweet potato via somatic embryogenesis using diverse explants anther (Tsay et al. 1979), leaf, internode, root (Yamaguchi et al. 1973; Sehgal 1975; Liu et al. 1984; Sefasi et al. 2012), petiole (Liu et al. 1985), lateral bud (Tang et al. 1993; Cavalcante et al. 1994; Desamero et al. 1994; Al-Mazrooei et al. 1997) and apical meristem of sweet potato (Jarret et al. 1984; Chee et al. 1988, 1988; Chee et al. 1989; Liu et al. 1989; Otani et al. 1996; Lee et al. 2006; Kwon et al. 2002) have not been successful, in part because of its low frequency of somatic embryogenesis. In contrast, the majority of successful plant regeneration results were obtained by using an apical meristem tissue culture which is different from embryogenesis, and being exploited for plant transformation.

Although sweet potato has high economic value and the potential to solve environmental problems and food problems, its productivity is constrained by the difficult induction of somatic embryogenesis. Therefore, this study was conducted to develop a protocol for induction of high frequencies of somatic embryo calli that can be useful in plant regeneration of sweet potato. Furthermore, this one-step plant regeneration system skipping a sensitive hormone shift process paves the way for improved breeding of sweet potato.

Materials and Methods

Plant materials and sterilization of tissues

Sweet potato cultivars ‘Sinwhangmi’, ‘Ginhongmi’, ‘Sinyulmi’, ‘Yunmi’ and ‘Sinzami’ used in this study were provided by the National Institute of Crop Science and cultivated at the Kyungpook National University farm and Gunwi farm. The internodes and leaves of 3~4 week old sweet potato plants were surface-sterilized by placing them in 50ml triangular flasks containing 70% ethanol for 15 sec, followed by rinsing with sterile water three times (30 sec, 1 min and then 2 min). And the tissues were treated with 3% NaOCl (8 min for leaves, 9 min for internodes), sterile water three times (30 sec, 1 min and then 2 min), Tween 20 for 30 sec and then sterile water three times (50 sec, 1 min and then 2 min).

Culture method and plant regeneration

The tissues were blot dried on sterile filter paper for 5 min, after which ten tissues were sown in 90×15 mm petri dishes containing 25ml of MS medium (Murashige et al. 1962). To establish an optimum condition of regeneration media, the 22 combinations of plant hormones by using three different classes of auxins (2,4-D, NAA, IAA) and cytokinins (BA, zeatin, kinetin) were formulated (Table 1). All agar was solidified with 8g/L plant agar (Duchefa Biochemie, Haarilem, The Netherlands) and 30 g/L sucrose (Duchefa Biochemie) and the pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 18 psi for 20 min. Ten segments of each tissue (young leaf, leaf disc, internode or petiole) were placed on the medium in petri dish, and there were five replicates for each test medium and tissue type.

Table 1. Composition of plant growth regulator in MS medium

Medium number	Medium	Auxin (mg/L)			Cytokinin (mg/L)		
		2,4-D	NAA	IAA	BA	Zeatin	Kinetin
1	MS						
2						2.0	
3				0.5	1.0		
4				0.5	10.0		
5			0.1		2.0		
6			0.3			2.3	
7			1.0				
8			1.0		5.0		
9			2.0				
10		0.05					
11		0.05					0.5
12		0.1					
13		0.2	0.5			0.5	
14		0.5					
15		0.5	0.5				
16		1.0					
17		1.0			2.0		
18		1.0	1.0				
19		1.0	2.0				
20		2.0					
21		2.0	1.0				
22		2.0	2.0				

Tissues from plants were dissected and subsequently placed into the same media in either the normal vertical direction or the opposite direction. The petri dishes were incubated in dark condition to induce the formation of calli. Petri dishes were incubated for 5 days at 27 °C under a 24-h dark condition. After 5 days, petri dishes were incubated for 4 months at 27°C under a 16/8-h (day/night) photoperiod. The induced callus was subcultured on the fresh media in every 2~4 weeks for further development. The following plant regeneration acclimation method was used to generate plants from the leaves and roots. The cultured tissue was first rinsed with 20°C distilled water and treated with 4% Hyponex solution for acclimation. The sample was then transferred to a growth chamber and cultivated at 30°C under a 16/8-h (day/night) photoperiod for five days in a sealed bottle with a hole in the seal. After 5 day, plants were transferred to a soil pot and placed in a growth chamber at 30°C under a 16/8-h (day/night) photoperiod.

Results

Samples were sterilized using sodium hypochlorite at 3%, 5% or 7% and then checked every fourth day. The percentage of disinfected tissues 4 days after culture at the hypochlorite concentration of 3%, 5%, and 7% was 18.4%, 14.6%, and 16.6%, respectively. The optimum results were observed when 3% was used; therefore, this concentration was used for subsequent procedures. The greatest callus induction rate was observed for MS supplemented with 1.0 mg/L 2,4-D in the ‘Ginhongmi’ and ‘Sinwhangmi’ cultivars; therefore these culture condition was applied for the subsequent experiments (data not shown).

In this study, there were two types of calli formed, hard featured (easy to break) or soft one (slurry). The majority of calli formed on MS medium containing 1.0 mg/L NAA and 0.5 mg/L BA were hard and difficult to break. In contrast, MS medium containing 1.0 mg/L 2,4-D or 2,4-D with NAA resulted in generation of calli that were friable and festered. Hard and easy to break calli were formed on MS medium containing cytokines (BA, zeatin, kinetin) and auxins (2,4-D, NAA, IAA).

The root formation frequency from the internode cultivated on MS with 1.0 mg/L NAA and 5.0 mg/L BA, MS with 0.1 mg/L NAA and 2.0 mg/L BA, MS with 0.3 mg/L NAA and 2.3 mg/L zeatin and MS with 1.0 mg/L NAA were 85%, 74%, 50% and 50%, respectively. In addition, the root formation frequency from the leaf disc cultivated on MS with 1.0 mg/L NAA, MS with 0.3 mg/L NAA and 2.3 mg/L zeatin, MS with 0.05 mg/L 2,4-D, and MS with 10.0 mg/L BA and 0.5 mg/L IAA were 80%, 65%, 45% and 33%, respectively. The plant regeneration rates from the tissue of internode and young leaf in MS medium supplemented with 1.0 mg/L NAA and 5.0 mg/L BA were 35% and 4%, respectively.

There is currently a need for development of new varieties of sweet potatoes owing to environmental pollution, changing natural environments and consumer demand. Previous experiments investigating regeneration tissue culture of sweet potato used apical meristems. However, in this experiment leaf discs, whole leaves, internodes and petioles were used.

Calli formed under dark conditions were white; however, some became yellow after transfer to light conditions. Embryogenic calli were compact and light green or bright yellow, while non-embryogenic calli were white and friable and formed earlier and faster than embryogenic calli (Cipriani et al. 1999) (Table 2).

Table 2. Callus color and physical properties of growth regulators in MS medium.

Medium number[a]	Internode		Whole leaf	
	Callus color	Callus hardness	Callus color	Callus hardness
1	W(G)	H	W(G)	H
2	W(G)	H	W(G)	H
3	W(W)	H	W(W)	H
4	W(G)	H	W(G)	H
5	W(G)	H	W(G)	H
6	W(G)	H	W(W)	H
7	W(G)	H	W(G)	H
8	W(G)	H	W(G)	H
9	W(G)	H	W(G)	H
10	W(G)	H	W(G)	H
11	W(G)	H	W(G)	H
12	W(W)	H	W(G)	F
13	W(G)	H	W(G)	H
14	W(Y)	H	W(Y)	F
15	W(Y)	H	W(Y)	F
16	W(Y)	F	W(Y)	F
17	W(W)	H	W(Y)	H
18	W(Y)	F	W(Y)	F
19	W(Y)	F	W(Y)	F
20	W(Y)	F	W(Y)	F
21	W(Y)	F	W(Y)	F
22	W(Y)	F	W(Y)	F

[a]Medium number is the same as in Table 1. Explant culture: each concentration 50 tissues, ( ) : Changed callus color in light condition.

From the internodes, the highest plant regeneration and root formation rates were observed in MS medium supplemented with 1.0 mg/L NAA and 5.0 mg/L BA. For young leaves, the highest plant regeneration was occurred in MS medium with 0.1 mg/L NAA and 2.0 mg/L BA, and the root formation was in MS with 1.0 mg/L NAA. In the combination of 0.1 mg/L NAA and 2.0 mg/L BA, both young leaves and leaf discs were developed in shoot and root (Table 3).

Different combination of plant hormones also affected the color of calli. In case of internode tissue, MS medium with 0.5mg/L IAA and 1.0 mg/L BA produced greenish calli, while in MS with 1.0 mg/L2,4-D and 2.0 mg/L BA produced normal white calli. In addition, MS medium supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L NAA generated calli in yellow (Fig. 1C).

In internode and young leaf, MS medium with 0.3 mg/L NAA and 2.3 mg/L zeatin was optimal hormonal condition for root formation, and in internode MS with 1.0 mg/L BA and 0.5 mg/L IAA root formation (Fig. 2D). In MS medium with 0.5 mg/L 2,4-D and 0.5 mg/L NAA generated root formation. In MS with 1.0 mg/L NAA and 5.0 mg/L BA medium efficiently produced plant regeneration. After either shoot or root was formed from the calli, eventually those tissues were further developed to a whole plantlet without subculturing on hormone-free media (Fig. 2D). Internode tissue remained on samples cultivated in MS medium with 1.0 mg/L NAA and 5.0 mg/L BA, induction callus and callus were regeneration (Fig. 2E). Young leaves remained on samples cultivated on MS medium supplemented with 0.1 mg/L NAA and 2.0 mg/L BA, induced calli and further developed to a whole plantlet.

There was a regeneration plant survival rate on 4% Hyponex culture solution of 97.6%, while the survival rate of those transferred to the pot was 92.7%. The survival rate of plants formed from regenerated roots on 4% Hyponex culture solution was 84.8%, while that of those transferred to pots was 82.1 (Fig. 2F, Table 4).

Micropropagation starts with the selection of explants from a healthy, vigorous mother plant. Any part of the plant (leaf, apical meristem, bud and root) can be used as an explant. The entire process can be summarized into the following stages (Fig. 2F). Preparation of donor plant can be made using any plant tissue. This is followed by the initiation stage, when the explant is surface sterilized and transferred into nutrient medium. The plant subsequently enters the multiplication stage, which is meant to increase the number of propagules. The rooting stage may occur simultaneously in the same culture media used for multiplication of the explants. However, in some cases it is necessary to change media to induce rooting and the development of strong root growth. During the acclimatization stage, the *in vitro* plants are weaned and hardened. Hardening is conducted gradually from high to low humidity and from low light intensity to high light intensity. The plants are then transferred to an appropriate substrate (sand, peat, compost, etc.) and gradually hardened under greenhouse conditions. This process usually involves two culture steps, but one culture step was used in the present study (Fig. 3).

Table 3. Frequency of root formation and plant regeneration under light conditions

Medium number	Internode		Young leaf	
	Shoot formation (%)	Root formation (%)	Plant regeneration (%)	Root formation (%)
1	0	0	0	0
2	0	0	0	0
3	0	20	0	0
4	0	25	0	33
5	22	74	4	20
6	0	50	0	65
7	0	50	0	80
8	35	85	0	2
9	0	33	0	0
10	0	30	0	45
11	0	2	0	0
12	0	0	0	0
13	0	0	0	0
14	0	0	0	0
15	0	32	0	0
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	0	0	0	0
22	0	0	0	0

Table 4. Survival rate in pots, regeneration of plants through an acclimation process

Population Number (A, No.)		Acclimation population number of plants (B, %)	Plant survival rate in pot (C, %)
Plant	Root		
42		41(97.6)	38 (92.7) C/A*100[90.5%]
	33	28(84.8)	23 (82.1) C/A*100[69.7%]

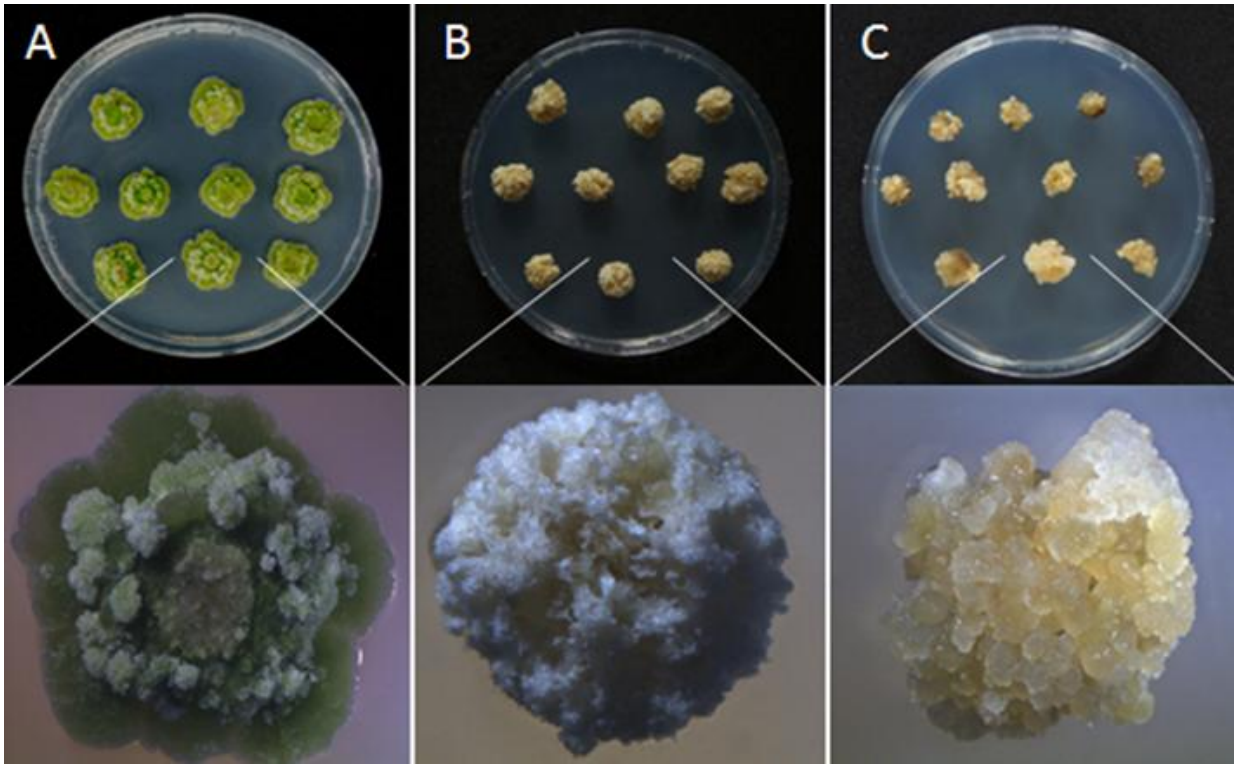

Fig. 1. The features of callus formation under different plant hormone condition. A: MS with 0.5mg/L IAA and 1.0 mg/L BA, B: MS with 1.0 mg/L2,4-D and 2.0 mg/L BA, C: MS with 0.5 mg/L 2,4-D and 0.5 mg/L NAA.

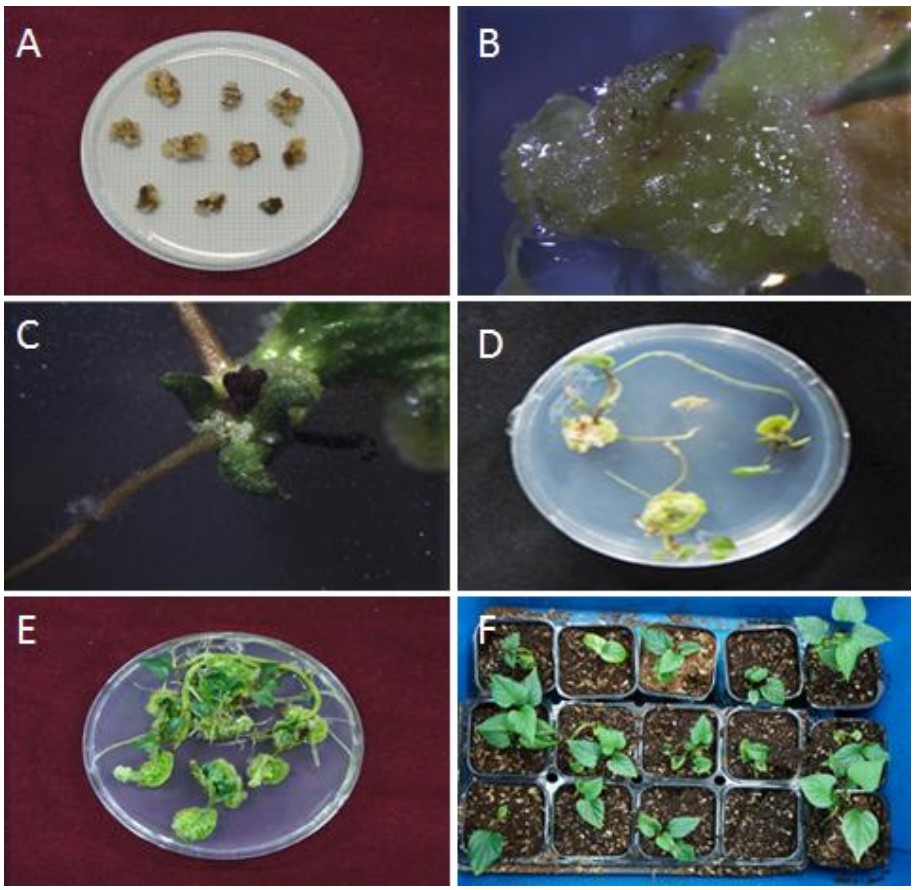

Fig. 2. Plant regeneration in MS medium with 0.1 mg/L NAA and 2.0 mg/L BA. A : callus formation among cultivars of sweet potato in the medium condition of MS supplemented with 1.0 mg/L and 2,4-D, B : green spot regeneration in MS medium with 0.1 mg/L NAA and 2.0 mg/L BA, C : shoot and rooted from green spot, D : plant lets on the plant regeneration medium, E : young plant on the plant regeneration medium, F : regenerated plants in pots controlled in growth chamber.

Regeneration plant survival rate after acclimation in pots was 90%, which is very high. Additionally, the highest induction of calli, roots and plant regeneration was observed on MS medium containing NAA and BA. However, others studies have shown the best embryogenic response from medium containing 2,4-D (Sefasi et al. 2012; Cipriani et al. 2001; Sihachakr et al. 1997), although 2,4,5-T has been reported to be better than 2,4-D for inducing embryogenic calli (Al-Mazrooei et al. 1997; Triqui et al. 2008). Successful regeneration of transgenic plants from five Japanese cultivars has also been reported when 4-FA was used, although there was no success when 2,4-D was used (Anwar et al. 2010).

Low numbers of regenerated plants have been reported in many previous studies of sweet potato cultivars from different geographical areas (Otani et al. 2003). The repetitive regeneration of cultivars Huachano, Jonathan and Jewel in a short time through somatic embryogenesis in this study is a critical breakthrough, although some researchers regenerated these popular cultivars previously. It is difficult to reproduce regeneration results from one experiment to the next or from one laboratory to another. Moran et al. (Moran et al. 1998) reported that many protocols that had previously been reported to lead to plant regeneration did not produce good results in their laboratory. The inconsistencies in regeneration responses within the same cultivar may be due to variations in the developmental and physiological stage of *in vitro* plants, which affect the cultural behavior of explants (Triqui et al. 2008; Hones et al. 2007). For example, the sweet potato cultivar Duclos 11, which previously showed the ability to regenerate plants from protoplast-derived calli (Sihachakr et al. 1987), showed no embryogenic response when lateral buds were used later (Sihachakr et al. 1997). It is important to first establish a system for somatic embryogenesis for the recalcitran cultivar to facilitate regeneration and enable application of genetic engineering to improve important traits. Yang (Yang et al. 2011) reported formed embryogenic callus by apical and axillary buds of sweet potato, and improved transformation efficiency to use this. But, our result did to do plant regeneration forming embryogenic callus to use formation that meristem of sweet potato is not, and shortened very at the time. In conclusion, this study elucidated the optimum hormonal condition and the most effective explant source for the one-step plant regeneration system via somatic embryogenesis in sweet potato. Moreover, from the assay has identified in plant regeneration of the yarkon which is tuberous roots as the sweet potato. The highest callus induction and plant regeneration was observed on MS medium containing BA and 2,4-D, NAA and 2,4-D, or BA and NAA (Cynthia et al. 2009; Liu et al. 2009).

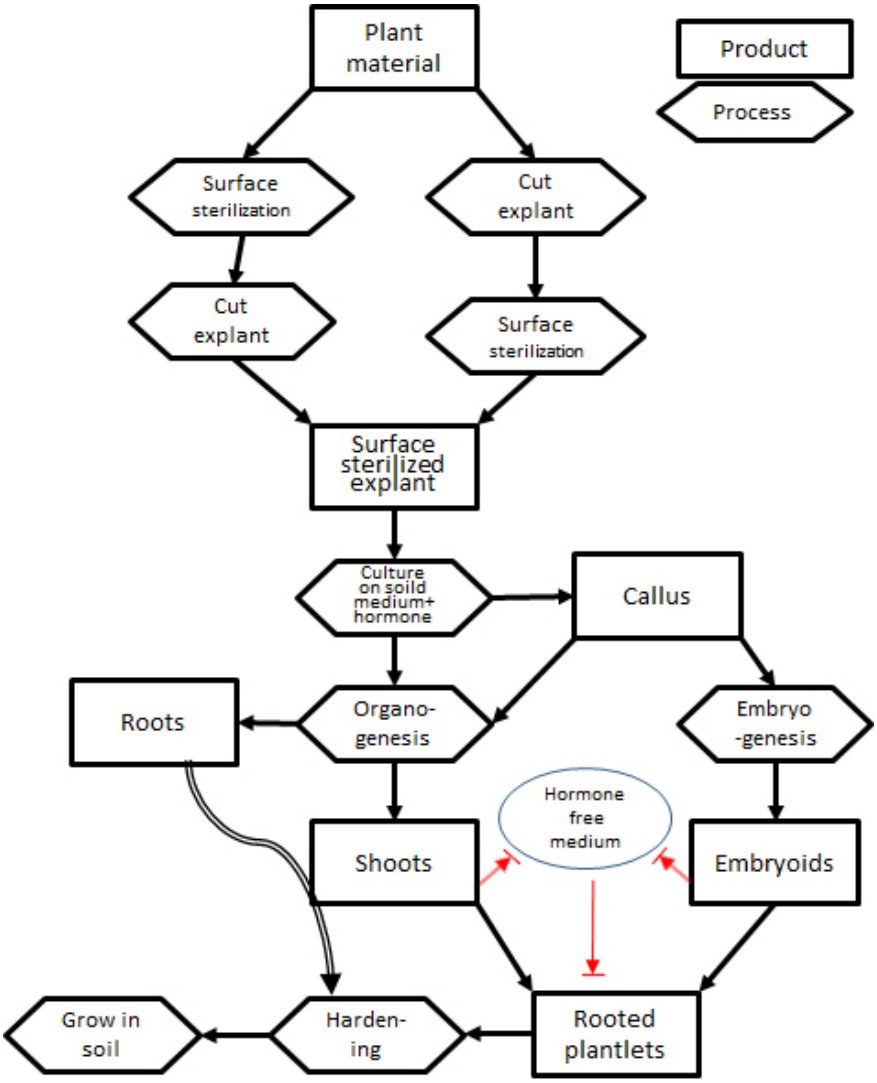

Fig. 3. Flow chart of the one-step plant regeneration system through diverse processes including organogenesis and embryogenesis. Generally, shoots or embryoids formed on hormonal media are required to be transferred to hormone-free media to produce a whole plantlet. In this study, we successfully regenerated sweet potato plants in the initial hormone media without subculture. Red blocked lines indicate that the subculture step to hormone-free media is bypassed, thus it allows one-step plant regeneration system. Furthermore, a whole plantlet was regenerated even from roots (double line) with high frequency (69.7%). This new approach might pave the new way for the highly efficient regeneration system in plants including sweet potato.

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