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High-frequency plant regeneration through secondary somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq. *var.* tenera)

S. Te-chato[*] and A. Hilae

Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

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A high-frequency plantlet regeneration protocol was developed for oil palm (*Elaeis guineensis* Jacq. *var* tenera) through secondary somatic embryogenesis. Secondary embryos formed from the basal end the primary haustorium embryos (PHE) which were originally derived from coleoptile tissues of germinating PHE on MS medium supplemented with 0.1 mg/l dicamba, 200 mg/l ascorbic acid in the presence of 0.2 M sorbitol. The process of secondary embryogenesis continued in a cyclic manner from the shoot pole of newly formed embryos resulting in clumps of somatic embryos. Strength of the medium and kinds and concentrations of sugar influenced the process of secondary embryogenesis and germination of somatic embryo. Full-strength MS medium supplemented with 0.2 M sorbitol produced significantly higher percentage and numbers of secondary somatic embryos and further development of proliferated embryos into plantlets. The system of secondary somatic embryogenesis in oil palm described here represents a permanent source of embryogenic material that can be used for genetic manipulations of this crop species.

Key words: embryogenesis, oil palm (*Elaeis guineensis* Jacq. *var*. tenera)

Introduction

Oil palm is the most efficient oil-bearing crop in the world with an average yield of 4 - 5 tonnes of crude oil per hectare and up to 7 - 8 tonnes of crude oil per hectare. As a comparison, palm oil can yield 5950 L oil/ha, approximately 5 times that of olive oil (1212 L oil/ha) and 13 times that of soybean (446 L oil/ha) (Biofuel, 2007). Indeed, the large amount of oil produced in the oil palm fruit is unique biological characteristic of this palm species. For Thailand, the oil has been brought to production of biodisel. In the next five years (2011) the government have a policy to increase an area for oil palm planting to 10 million rai (1.6 million acre). In this activity a high yield or superior tenera palms are needed for fuel oil/biodisel production.

[*] Corresponding author: Sompong Te-chato; e-mail: stechato@yahoo.com

Commercial propagation of oil palm through tissue culture is widely used (Khaw *et al.,* 1999). A working team from Malaysia has proved that clones obtained from this technique gave a far better yield than seeded plants (30%) (Khoo *et al.,* 1999). In their report it took at least 4 years to induce plantlet regeneration from original tissue. By the manipulation of phytohormones and carbon sources Te-chato *et al.* (2002) could shorten this time to more than half.

Secondary somatic embryogenesis is a process whereby new somatic embryos are initiated from originally formed somatic embryos or primary somatic embryos. As an experimental system it has certain advantages compared to primary somatic embryogenesis such as very high multiplication rate, independence of an explant source and repeatability. Additionally, embryogenicity can be maintained for long period of time by repeated cycles of secondary embryogenesis. Furthermore, in many species the efficiency of explants in primary embryogenesis is lower than in secondary embryogenesis. This phenomenon have been described in at least 80 Gymnosperm and Angiosperm species (Raemakers *et al*., 1995). High-frequency plant regeneration systems through secondary embryogenesis were reported in several plant species of interest (Merkle *et al.,* 1990; Raemakers *et al.,* 1993a, b; Weissinger II and Parrott, 1993; Choi *et al*., 1997; Das *et al*., 1997; das Neves *et al.,* 1999; Chen and Chang, 2004; Giridhar *et al.,* 2004).

Somatic embryogenesis (SE) in oil palm has been documented from young leaf-(Te-chato *et al*., 1998a; Te-chato *et al*. 1998b), zygotic embryo-derived callus (Nwanko and Krikorian, 1983; Rabechault and Cas, 1974) and directly from young leaves from fond number 6-8 after third subculture (unpublished data). The ability of oil palm zygotic embryos to produce secondary embryos has also been indicated (Rajesh *et al.,* 2003). However, percentage and numbers of new forming embryos were limited and germination of those embryos were not reported. In preliminary study of Hilae and Te-chato (2005), induction secondary somatic embryogenesis using primary somatic embryos was feasible. So, the present study reports the establishment of a high-frequency plant regeneration system of oil palm through secondary embryogenesis.

Material and methods

Establishment of primary somatic embryogenic cultures

Primary embryogenic cultures were established following the method described by Hilae and Te-chato (2005). Primary callus (PC) induced from leaf segments according to was regularly transferred to fresh MS medium

supplemented with 1 mg/l Di, 200 mg/l ascorbic acid (designated MS-P medium) 4-week intervals. The cultures were placed under light conditions (2,500 lux, 14 hour photoperiod) at 24-30^{o}C.

Induction of secondary somatic embryogenesis (SSE)

PHEs were carefully detached and inoculated on one-fifth or half- or full-strength, PGR-free MS medium containing sucrose or glucose or sorbitol 0.1, 0.2 or 0.3M and gelled with 0.65% agar-agar (Bacteriological grade, Hi-media). The pH of the medium was adjusted to 5.8 prior to autoclaving. Cultures were maintained at 2,000 lux (14 hour photoperiod) at a temperature of 28±0.5°C. The culture conditions remained the same for all further experiments unless otherwise specified. While inoculating, the PHEs were uniformly spread on the surface of the medium. There were 4 replicates (Borosil culture tubes of 25×150 mm containing 10 ml of medium) each containing 25 PHEs per replicate. The percentage of cultures that produced secondary somatic embryos (SSE) and number of SSE per tube were recorded after 180 days of culture by counting under a stereo-zoom microscope (Nikon, SMZU).

Germination of Secondary somatic (SSE)

SSE together with haustorium-staged embryos from those sugars containing medium were transferred to growth regulator-free MS medium solidified with 0.7% agar and adjusted pH to 5.7 before autoclaving. All cultures were carried out in 25x150 mm culture bottle under 14 h photoperiod, 1,300 lux illumination. After 4 months (observed monthly interval) germination percentage and number of seedlings/SSE clump were recorded.

Results and discussion

Establishment of primary somatic embryogenic cultures

Initially, the cultures have to started from suitable explant and plant growth regulators. In general, primary somatic embryo from young leaves of oil palm is indirect and initiated by auxin, 2,4-dichlorophenoxy acetic acid (2,4-D) (Khoo *et al.*,1999) or dicamba (Di) (Te-chato, 2002). Within 16 wk of culture, various stages of somatic embryos from globular to haustorium (mature somatic embryo) were obtained. In some culture, primary haustorium

Table 1. Effect of kind and concentration of sugar and strengths of MS medium on SSE formation after culturing for 3 months.

Carbon Source	Stength of MS	Concentration (M)	SSE formation	
			Number	%
Sucrose	1	0.1	22.0	10.09
		0.2	15.40	10.38
		0.3	7.80	14.23
	1⁄2	0.1	10.60	24.52
		0.2	10.10	14.54
		0.3	11.44	17.48
	1⁄5	0.1	6.60	24.24
		0.2	4.00	25
		0.3	6.20	17.74
Fructose	1	0.1	6.67	44.97
		0.2	2.4	54.16
		0.3	3.8	44.73
	1⁄2	0.1	7.0	32.85
		0.2	5.2	36.53
		0.3	1.1	90.9
	1⁄5	0.1	3.2	62.5
		0.2	2.9	44.82
		0.3	1.0	100
Glucose	1	0.1	16.67	38.63
		0.2	17.0	11.05
		0.3	19.3	12.43
	1⁄2	0.1	14.6	21.9
		0.2	17.25	7.97
		0.3	8.8	13.6
	1⁄5	0.1	6.22	23.15
		0.2	7.0	27.71
		0.3	15.2	7.23
Mannitol	1	0.1	0	0
		0.2	0	0
		0.3	0	0
	1⁄2	0.1	0	0
		0.2	0	0
		0.3	0	0
	1⁄5	0.1	0	0
		0.2	0	0
		0.3	0	0

Table 1. Continue..

Carbon Source	Stength of MS	Concentration (M)	SSE formation	
			Number	%
Sorbitol	1	0.1	11.55	21.11
		0.2	21.55	100
		0.3	2.22	45.04
	1/2	0.1	11.16	41.75
		0.2	3.71	46.09
		0.3	5.12	58.59
	1/5	0.1	7.25	27.58
		0.2	3.71	46.09
		0.3	4.99	31.11

somatic embryos (PHEs) derived from PC after 12 wk of culture. In general, time consume for embryogenic callus induction of oil palm has been reported to be more than one year (Khoo *et al.*,1999). In cassava, somatic embryogenesis starts with the culture of leaf explants on solid Murashige and Skoog-based medium supplemented with auxins and mature somatic embryos are formed within 6 wk (Raemakers *et al.*, 1993a, b). Sources of explant for induction of SSE were differed from species to species. The cotyledons of the primary somatic embryos were proved to be a good explants for a new cycle of somatic embryogenesis. The cotyledons undergo secondary somatic embryogenesis on both liquid and solid Murashige and Skoog-based medium supplemented with auxins within 13-40 days (Raemakers *et al.*, 1993b). Immature male inflorescences of banana was proved to be a good starting explant for the regeneration of plants via secondary somatic embryogenesis (SSE). Since new embryos are continually formed from existing embryos, SSE has the potential to produce many plants and, once initiated, may continue to produce embryos over a long period of time (banana). In oil palm, zygotic embryos was tried but small number of SSE induced (Rajesh *et al.*, 2003). In this present study, PHEs were of great important for utilization in inducing SSE.

Induction of SSE

SSE is the process of induction of new somatic embryos from pre-existing embryos (Raemakers *et al*., 1995). Since new embryos are continually formed from existing embryos, SSE has the potential to produce many plants and, once initiated, may continue to produce embryos over a long period of time. SSE is a new cycle of somatic embryogenesis which could be induced directly from PHE. For strengths of MS medium full strength supplemented

with 0.1 mg/l dicamba in the presence of all sources of sugars gave the best result in SSE formation. Among carbon sources tested, 0.2M sorbitol resulted in the best SSE induction both percentage (100) and number of SSE (21.55 SSE/PHE) while all concentration of mannitol gave no response in SSE formation (Table 1, Fig. 1). Even sucrose yielded number of SSE slightly higher but percentage of SSE formation was far lower. Those SSEs were white, opaque and torpedo in shape (Fig. 1) which arisen directly from peripheral layer of basal part of PHE (Promchan and Te-chato, 2007). SSEs were also reported to be induced indirectly from SE-derived cotyledon in cotton (Raemakers *et al.*, 2000). In terms of chemical factor, not only plant growth regulators play role on SSE formation but sugars and polyamines also act as promoting substances. However, the key factors for inducing SSE depended upon plant species. For oil palm, 0.1 mg/l dicamba in combination with 0.2 M sorbitol proved to be the optimum for SSE production. Polyamine; spermine, spermidine has been reported to induce SSE from culturing zygotic embryo of oil palm (Rajesh *et al.*, 2003), unfortunately, low efficiency of SSE induction obtained. In banana, MS supplemented with 10% coconut water produced rapidly proliferating embryogenic callus that developed into secondary somatic embryos (SSE) (Khalil *et al.*,2002).

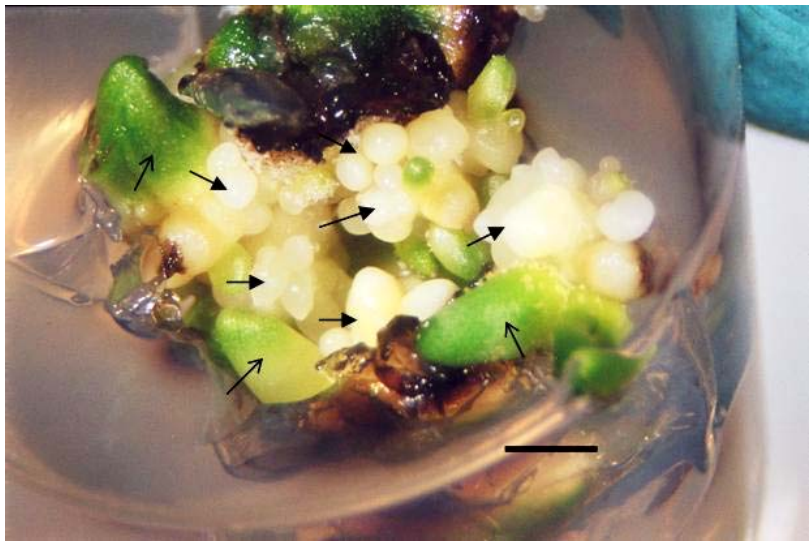

Fig. 1. Cluster of torpedo-stage secondary somatic embryos (⟶) at the basal part of PHE (⇒) on sorbitol containing medium. (*bar*: 1 cm)

Germination of embryoids

Typically, germination of embryoids is carried out by transferring various stages of embryoids to culture medium without PGRs. The removal of the auxin from medium suppressed newly forming embryos. However, this is not necessary in all cases. In oil palm normal germination of oil palm somatic

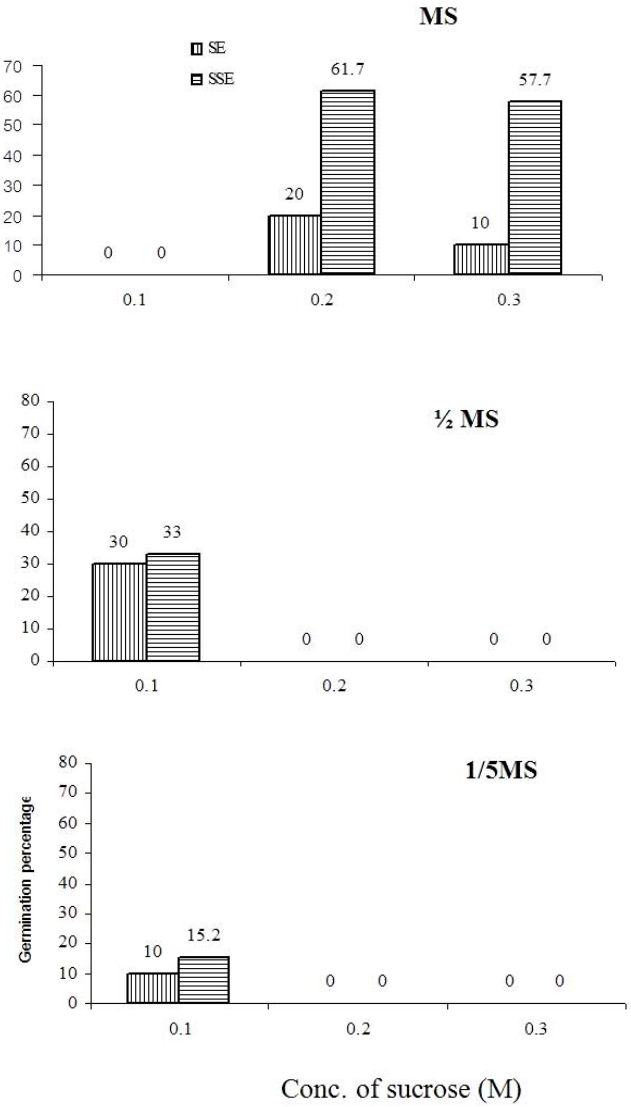

Fig. 2. Effect of concentration of sucrose and strength of MS medium on germination of SE and SSE.

embryo (SE) developed normal seedlings in liquid MS or ½MS mediuin in the presence of low concentration of NAA (0.06 mg/l) and BA (0.03 mg/l) (Techato and Muangkaewngam, 1992). However, low efficiency of germination obtained. By modification of sugars and plant growth regulator in various strength of MS medium it was clear evident that SEE formed from the basal

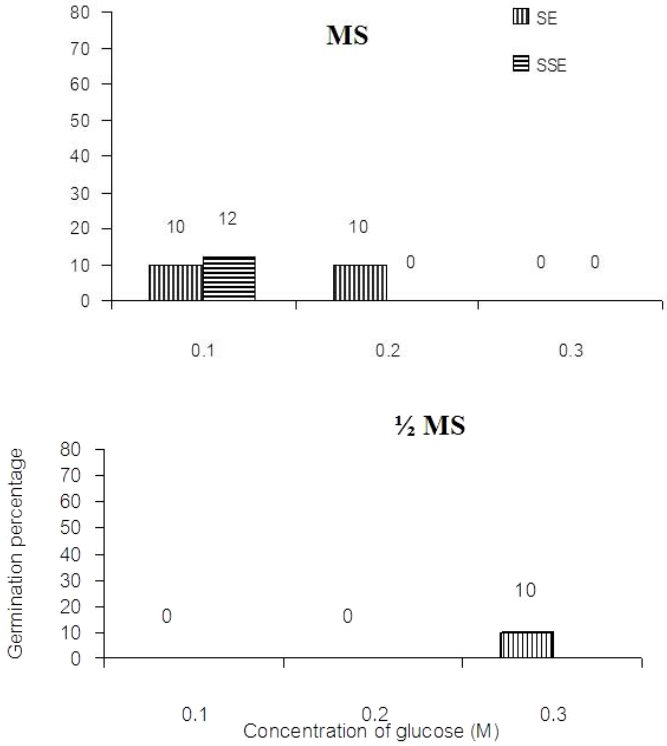

Fig. 3. Effect of different concentrations and glucose and MS medium on germination percentage of SE and SSE.

part of SSE could germinate on PGR-free medium (Fig. 2-5). SSE induced on 0.2 M sorbitol and 0.1 mg/l dicamba containing full strength MS medium germinated as seedlings (both shoot and root) or shoot at the highest percentage of 78 (Fig. 4). While SE could germinate at only half (40%) of SSE. Mature SSE possessed a well-defined whitish opaque torpedo-stage morphology with a cylindrical, distinct apical dome with leaf primordia and crown region from which shoot initials and root initials developed (Fig. 5A). Embryos that germinated with shoot initials and root initials produced plantlets when placed directly on PGR-free medium. On this medium, which contained

no plant growth regulators, SSE gave rise to small plantlets within 30-45 days (Fig. 5B). The use of SSE could provide an efficient solution to the problems limiting plant regeneration in oil palm like those reports in banana cultivars (Khalil *et al.*, 2002). The detail protocol of SSE induction was shown in Fig. 6. This also suggested that SSE are probably of unicellular origin, making them an excellent candidate for genetic transformation since the potential for production of chimeric plants is low. Although the regeneration of oil palm through SSE has been previously reported (Rajesh *et al.*, 2003), these protocols are of great potent.

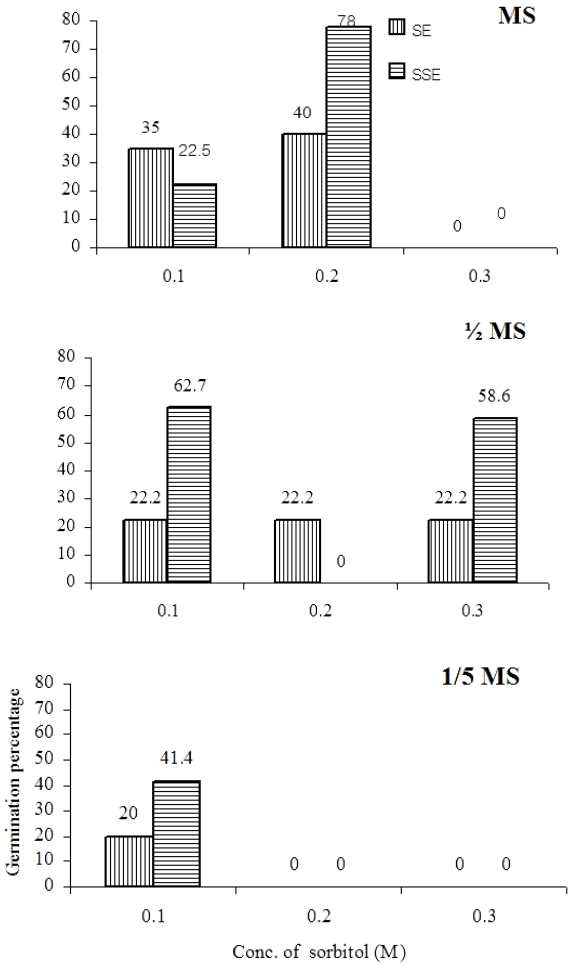

Fig. 4. Effect of concentrations of sorbitol and strength of MS medium on germination percentage of SE and SSE.

The major difficulty in converting embryos into plants appears to be that the embryos are not actually mature, a stage characterized by accumulation of embryo-specific reserve food materials and proteins and by desiccation tolerance. In banana, mature somatic embryos germinate into plants after desiccation or dehydration and culture on a Murashige and Skoog-based medium supplemented with benzylaminopurine (BA) (Khalil *et al.,* 2002). Alcohol sugar or polyol e.g. polyethylene glycol (PEG) acts as osmotic stress and has a function like desiccation has been reported affecting on maturation and synchronization of the embryos (Mamiya and Sakamoto, 2000). In this present study, PEG was not tried but sorbitol enhanced both SSE formation and germination of them in the following step. High levels of sucrose (10% vs 3% in other media) have been reported to enhance normal maturation in somatic embryos include the addition of mannitol (4%) to the medium but failed to promote maturation and germination in oil palm. Those factors mimic the developmental environment of zygotic embryos and should further improve the maturation of somatic embryos.

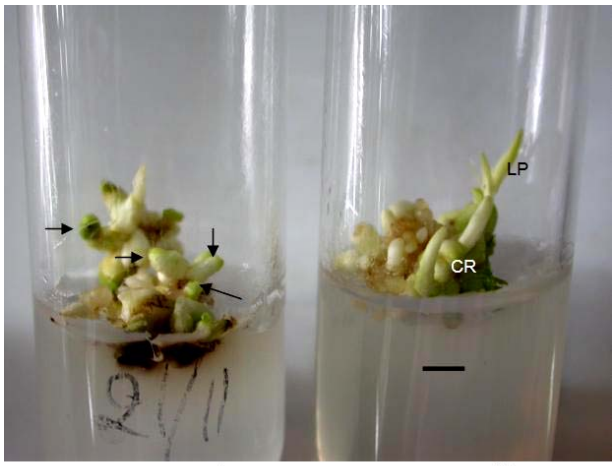

Fig. 5. Late torpedo-stage SSE on PGR-free MS medium (*bar*: 1 cm), A) green plumules (*arrow*) emerging, B) mature embryo with leaf primordia (*LP*) and crown region (*CR*).

In conclusion, the present study successfully describes the establishment of SSE of a cultivar of oil palm and demonstrates the potential of the system for scale-up indicating direct origin of SSE apparently from the maternal tissue. However, genetic fidelity of the regenerated plants and the origin of the SSE from the maternal tissue are to be confirmed through RAPD or AFLP.

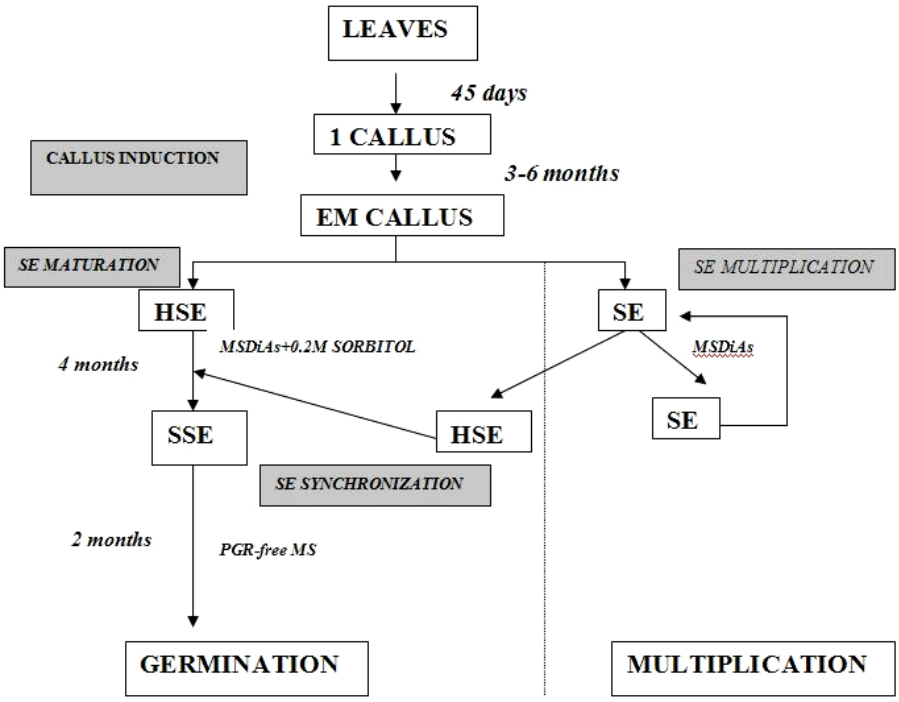

Fig. 6. Protocol for clonal propagation of oil palm through SSE.

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