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Plantlet regeneration via somatic embryogenesis from mature seed of *Citrus nobilis* variety SoE from SoE NTT

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ABSTRACT

The aim of this research is a study about direct somatic embryogenesis from mature seed and plantlet regeneration of *Citrus nobilis* variety SoE. Plant material for somatic embryogenesis is the mature seed of Tangerine SoE. 6-benzylaminopurine (BAP) 3 mg/L supplemented to Murashige and Skoog (MS) modified medium was induced direct somatic embryos (SE) 4 weeks after treatments. MS modified medium with single BAP 3 mg/L has resulted the average of eight SE. Nevertheless, average number of SE which resulted from combination BAP 3 mg/L with some level of concentration 2.4-D (0.1, 0.3, and 0.5 mg/L) decreases with increasing the concentration of 2.4-D. Abscisic acid (ABA) is added at concentration 0.5-3.5 mg/L cannot increase the number of SE, globular early-stage, at 4 and 8 weeks. Gibberellic acid (GA_3) added at concentration 2.5 mg/L can increase the growth in the number of normal plantlets this citrus at 8 weeks. Media of enlargement of Tangerine SoE plantlets used half-strength MS modified medium concentration. This medium can increase the average in height of plantlets at 4 weeks and the average in a number of leaves at 8 weeks. The regenerated plants were acclimatized under the 60% sunlight block and to keep the humidity remains high the plants were lid with UV plastic. The results indicated that the plant regeneration via somatic embryogenesis from mature seed of *C. nobilis* an efficient and effective technique for producing more SE and plant production in large scale. The next sub-cultured in medium containing GA_3 and then the half-strength concentration of medium can increase the number of normal and enlargement plantlets, respectively.

KEY WORDS: 2.4-D, 6-benzylaminopurine, gibberellic acid, regeneration, somatic embryo, Tangerine SoE

INTRODUCTION

Citrus nobilis variety SoE (Tangerine SoE) is a specific citrus from Mutis mountain region, 2000 m from sea level (BPTP-NTT), district of SoE, regency of Timur Tengah Selatan (TTS), Province of Nusa Tenggara Timur (NTT). This kind of citrus was started to be developed and "try to" be planted outside the area of origin because it is different with orange flavor of kind. The pH 5-7 suitable for growth (Martosupono *et al*., 2007). The flesh of fruit has a distinctive flavor, sweet with a sour taste fresh, young fruit skin is green-yellow, old fruit skin is orange-red, round flat fruit-shape (in observed, 2013), the height of stem 2-4 m, fruiting age is 2-3 years, contains more than 170 different phytonutrients (BPTP-NTT).

One of the ways, which efficient and effective to *in vitro* multiple plants, is by somatic embryogenesis technique. Somatic embryogenesis is a procedure whereby a cell or group of the cell from somatic tissue forms an embryo. The expansion of somatic embryos (SE) nearly replicates the process of zygotic embryo formation. Somatic embryogenesis mostly occurs indirectly via a superseding callus phase or directly from early explants (Han *et al*., 2011). A simple procedure for highly efficient and rapid plant regeneration via somatic embryogenesis in cotton, more than 20 Chinese

and Australian commercialized cotton cultivar, and this procedure promise to facilitating the application of plant genetic engineering on cotton genetic improvement (Zhang *et al*., 2009). The other researches, which have examined about plant regeneration via somatic embryogenesis such as the highest level for somatic embryogenesis (direct and indirect) to produce SE in *Citrus limon* L. achieved in the Murashige and Skoog (MS) medium containing 500 mg/L malt extract + 50 g/L sucrose + 3 mg/L BA) (Gholami *et al*., 2013). In pineapple plants used 1.0 mg/L (4.44 μM) BA in MS medium to developed SE into shoots (Sripaoraya *et al*., 2003). The best results for callus induction and SE initiation achieved with 3% sucrose and the hypocotyl and cotyledon explants. The SE was obtained with DKW medium and supplemented with 4 mg/L 2.4-D and 1 mg/L thidiazuron (TDZ); and 1 mg/L 2.4-D and 0.5 mg/L TDZ (Sie *et al*., 2010). *In vitro* young leaf explants treated with 2 mg/L BA was more efficient in inducing direct SE and subsequent plantlets growth in a short time duration (8-10 months) (El Bar and Dawayanti, 2014).

Somatic embryogenesis and plant regeneration from undeveloped ovules from 10 kinds citrus plants may induce using growth regulator such as 6-benzylaminopurine (BAP) 3 mg/L MS medium combined with 500 mg/L malt extract and 50 g/L sucrose (El-Sawy *et al*., 2006). 2.4-D 0.1 mg/L was optimally effective for maximum induction of embryogenic calluses and SE per explant leaves and stem of lovage plant (Park *et al*., 2010); 1-2 mg/L 2.4-D may induce embryogenic calluses and SE from the root of greater celandine plant (Lee *et al*., 2011).

BAP alone found to be effective in multiple shoot induction from nucellar embryo explants in *Citrus limonia* (Jajoo 2010). Vikram *et al*. (2012) were supplemented 0.2-1.0 mg/L Gibberellic acid (GA_3) to MS medium for shoots elongation. Its medium supplemented with 0.6 mg/L GA_3 combined with 1.0 mg/L Zeatin showed the maximum percentage of enhancement of shoots elongation. Lee *et al*. (2011) were selecting mature SE at the cotyledons stage of development for conversion and shoot growth. The plantlets were subculture on half-strength MS salts and vitamin in the absence of growth regulator for 1 month. Then, the plantlets were transferring regenerated plants to pots. According to Husni (2010), on citrus Siam, the success of maturation process with additional ABA 0.5 mg/L to *in vitro* medium of MS and vitamin molecular weight (MW) (MW medium) was 99%, better than media MW with additional ABA 0, 0.1, and 0.3 mg/L. While in germination process, germination to be the plantlet in MW medium with additional GA_3 0.5 mg/L in the amount of 58%, better than three doses of treatment of GA_3 (0, 0.1, 0.3 mg/L).

The aim of this research is a the study about direct somatic embryogenesis from mature seed and plantlet regeneration of *C. nobilis* variety SoE from SoE NTT.

MATERIALS AND METHODS

Plant Materials and Inoculants

Plant material as an explant in the induction of somatic embryo (SE) is cotyledons from mature seed *C. nobilis* varieties SoE from the district of SoE, regency of TTS, Province of NTT, and Indonesia. Inoculant for maturation process was embryogenic callus and cotyledons-embryos stage for germination. Material for enlargement of plantlets was individuals of citrus plantlets of Tangerine SoE from *in vitro* culture.

Culture Media for Treatments

The composition of the base as the media *in vitro* treatment media composed of MS + vitamin MW (MS modified) + PGR (according to treatment) + 3% sucrose + 0.25% phytagel. The complete composition of the treatment media saw in Table 1.

Sterilization of Seed

The mature seed of citrus washed with a detergent solution and rinsed with running water until lather missing. Furthermore, the sterilized seeds soaked in a solution hypochloride 50% for 15 min in the laminar air flow cabinet and rinsed three times with sterile distilled water.

Preparation and Initiation of Explant and Inoculant of Mature Seed

Seed coat shelled using a scalpel and tweezers. Once peeled, the seeds then inoculated into media *in vitro* MS modified without growth regulators. Shoots that grow from the seeds was the shoots derived from zygotic embryos. Hypocotyl origin zygotic embryo(s) that have germinated cut from explant and discarded. Cotyledons remaining subcultured to treatment media for SE induction. Embryogenic callus maturation process obtained from the globular stage SE growth. Cotyledons stage SE germination process obtained from SE growth.

Culture Conditions

Two pairs of cotyledons for SE induction, two small clumps callus embryogenic for maturation process, two cotyledons SE-stage for germination process and two plantlets with two segments have cultured in media in jar bottle (Table 1 for media composition). Cultures have placed in an incubation room with temperature 20°C,

Table 1: The composition of treatment media of somatic embryo induction, regeneration and enlargement plantlets (per litter medium)

The use of media *in vitro*	Code of treatment media	Composition solid media treatment (per litter media)
Basal media	MS modified	MS+vitamin MW 1 mg/L
Media of induction of somatic embryo	ES1	MS+vitamin MW 1 mg/L+BAP
	ES2	MS+vitamin MW 1 mg/L+BAP+2.4-D
	ES3	MS+vitamin MW 1 mg/L+BAP
	ES4	MS+vitamin MW 1 mg/L+BAP
Media of somatic embryo maturation	Ma1	MS+vitamin MW 1 mg/L
	Ma2	MS+vitamin MW 1 mg/L+ABA 0.5 mg/L
	Ma3	MS+vitamin MW 1 mg/L+ABA 1.5 mg/L
	Ma4	MS+vitamin MW 1 mg/L+ABA 2.5 mg/L
	Ma5	MS+vitamin MW 1 mg/L+ABA 3.5 mg/L
Media of mature somatic embryo germination	G1	MS+vitamin MW 1 mg/L
	G2	MS+vitamin MW 1 mg/L+GA_3 0.5 mg/L
	G3	MS+vitamin MW 1 mg/L+GA_3 1.5 mg/L
	G4	MS+vitamin MW 1 mg/L+GA_3 2.5 mg/L
	G5	MS+vitamin MW 1 mg/L+GA_3 3.5 mg/L
Media of enlargement plantlets	A	MS+vitamin MW 1 mg/L (Full concentration [1])
	B	MS+vitamin MW 1 mg/L (half concentration [1/2])
	C	MS+vitamin MW 1 mg/L (quarter concentration [1/4])

ABA: Abscisic acid, GA_3: Gibberellic acid, MS: Murashige and Skoog, MW: Molecular weight, BAP: 6-benzylaminopurine

photoperiod 24 h. All observation performed 4 and 8 weeks after treatment.

Plantlets Acclimatization

Plantlets that have been aged 4 months after germination conducted acclimatization process. Plantlets removed from the culture bottle. Media that are still attached to the roots washed with water. Media acclimatization consists of a mixture of soil, rice husk, and coconut coir in the ratio 1:1:1. Medium wetted with water up to field capacity. The roots of plantlets then planted, covered with a plastic sheet and placed under the auspices of shade with light intensity of 60%.

Statistical Analysis

Every experiment designed by completely random design. The experiment of induction of SE consists of four treatments and four replicates per treatments, the experiment of SE maturation and germination consists of 5 treatments and 10 replicates per treatments, and experiment of enlargement of plantlets consists of 3 treatments and 10 replicates. The data obtained were processed using Statistical Tool for Agricultural Research Software Program. Results of analysis of variance (ANOVA), which shows the real value of different F test, continued with Duncan multiple test rate 5% (DMRT 5%).

RESULTS AND DISCUSSION

Induction of SE of *C. nobilis* Variety SoE

The results of ANOVA (Table 1) showed that effect of treatment media of induction of SE (ES1-ES4) significantly different to forming SE at 4 weeks after treatment. However, at 8 weeks after treatment, the number of SE was not significantly different between treatments. The results of DMRT level 5% showed that the treatment of single plant growth regulator (PGR) of BAP 3 mg/L (ES1 medium) produce much more SEs most, an average of 8 pieces SE in 4 weeks after treatment. ES1 treatment medium significantly different with ES2, ES3, and ES4 treatment media; ES2 medium significantly different with ES3 and ES4; while among ES3 and ES4 media was not significantly different (Table 2).

The average of number of SE formed on treatment media ES1 (BAP 3 mg/L) more than the average of number of SE formed on media ES2, ES3, and ES4. The number of SE formed at 8 weeks after treatment even though was not significantly different between treatment media, but showed that treatment media ES1 (BAP 3 mg/L) still more produced SE than another three treatment media (Figure 1).

Maturation and Germination of SEs of *C. nobilis* Variety SoE

Media treatment of maturation and germination contain ABA and GA_3, respectively (Table 1, the composition of media). Table 3 showed the results of ANOVA the effect of maturation media (Ma1-Ma5) to embryogenic callus forming SEs (green spot and diameter of callus; and the effect of germination media (G1-G5) to mature SEs (cotyledons) forming the plantlets, at 4 and 8 weeks after treatments.

ANOVA of variable green spot, diameter of callus, number of normal and abnormal plantlets at 4 weeks after treatment showed that results were not significantly

Table 2: Analysis of variance media effects induction treatment of somatic embryos (ES1-ES4) to the average number of somatic embryos; and DMRT 5% (4 and 8 weeks after treatment)

Treatments media of induction of somatic embryo			Variable of observation (weeks after treatments)	
Code of treatments medium	PGR combination (mg/L)		Number of somatic embryos	
	BAP	2.4-D	4	8
ES1	3.0	0	8.00[a]	20.00[a]
ES2	3.0	0.1	3.75[ab]	5.75[a]
ES3	3.0	0.3	0.75[b]	4.00[a]
ES4	3.0	0.5	0.25[b]	0.75[a]
F value			5.84*	2.41
Pr>F			0.0107	0.1181

*F value very significantly different at level 5%. The same letter is not significantly different at further test DMRT level of 5%. DMRT: Duncan multiple test rate, PGR: Plant growth regulator, BAP: 6-benzylaminopurine

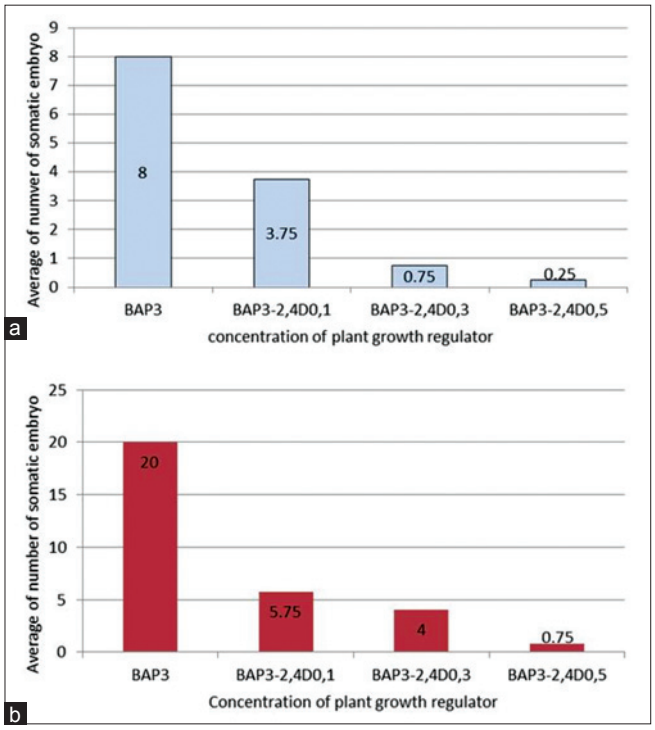

Figure 1: The average of number of somatic embryo formed on each induction treatment media. (a) 4 weeks, (b) 8 weeks after treatments

different from treatment media of maturation (Ma1-Ma5) and germination (G1-G5). While, observation at 8 weeks after treatment showed that the effect of maturation treatment media (Ma1-Ma5) was not significantly different of the number green spot and diameter of callus; for germination treatment media (G1-G5) the effect was significantly different of the number of normal plantlets, but it was not significantly different of the number of abnormal plantlets. Results of DMRT 5% effect each treatment media concentration (G1-G5) to the number of normal plantlets (Table 3). The effect of G4 treatment

Table 3: The analysis of variance (Anova) the effect of treatment media of maturation (ABA) and germination (GA_3) of somatic embryo; and DMRT5% (4 and 8 weeks after treatments)

Treatment media of maturation		Variable of observation (weeks after treatments)			
Media code	ABA concentration (mg/L)	The average of green spot		The average of diameter of calluses (cm)	
		4	8	4	8
Ma1	0	9.8	20.8	2.30	2.84
Ma2	0.5	6.4	14.3	2.20	2.71
Ma3	1.5	5.2	09.7	2.14	2.68
Ma4	2.5	5.6	10.7	2.12	2.58
Ma5	3.5	5.8	12.2	2.13	2.62
F value		0.77[ns]	1.38[ns]	0.21[ns]	1.33[ns]
Pr>F		0.55002	0.2548	0.9332	0.2736
Treatments media of germination		**Variable of observation (weeks after treatments)**			
Media code	GA_3 concentration (mg/L)	Number of normal plantlets		Number of abnormal plantlets	
		4	8	4	8
G1	0	1.41	1.15[b]	0.25	0.30
G2	0.5	1.00	1.10[b]	0.20	0.25
G3	1.5	1.00	1.25[b]	0	0.05
G4	2.5	1.50	2.50[a]	0.20	0.20
G5	3.5	2.00	2.30[a]	0.20	0.20
F value		0.62[ns]	10.91**	1.25[ns]	1.08[ns]
Pr>F		0.6533	0	0.3043	0.3783

**F value very significantly different at level 1%. The same letter is not significantly different at further test DMRT level of 5%. ABA: Abscisic acid, GA_3: Gibberellic acid, DMRT: Duncan multiple test rate

(2.5 plantlets) was not significantly different from G5 (2.3 plantlets). The effect of treatment medium between G4 and G5 to G1-G2-G3 was significantly different while G1-G2-G3 was not significantly different. The results showed that GA_3 with concentration 2.5 mg/L might stimulate the normal plantlet germination. However, G5 treatment media (3.5 mg/L GA_3) was not significantly different from G4 (2.5 mg/L GA_3) but the average of number of normal plantlets showed to decrease with increasing GA_3 concentration at G5.

Enlargement of Plantlets

Table 4 the results of ANOVA showed enlargement plantlets (A: Full, B: half, C: quarter concentration, Table 1, media composition) to the high difference plantlets, number of leaves, and number of roots, data were taken at weeks 4 and 8 after treatments.

The results of ANOVA (Table 4) showed that the effect of enlargement treatment plantlets media (media A, B, C) of the high variable plantlets (4 weeks after treatment) is highly significant. Number of leaves, number of roots (4 weeks after treatment) was not significant. Weeks 8 after treatment, media influence treatment A, B, C to the

Table 4: Analysis of variant influence of the media treatment of enlargement of plantlets (A, B, C) to the average difference in height of plantlets, number of leaves and root (4 and 8 weeks after treatment)

Code of media	Variable of observation (weeks after treatments)					
	The average of difference in height of plantlets (cm)		The average of difference in number of leaves		The average of difference in number of roots	
	4	8	4	8	4	8
A	0.0340^{b}	0.0770	0.43	0.77^{b}	0	-
B	0.1760^{a}	0.0840	1.40	1.53^{a}	0	-
C	0.1120^{a}	0.0870	0.53	0.83^{b}	0.067	-
F value	8.02**	0.19^{ns}	2.21^{ns}	5.18*	1.00^{ns}	-
Pr>F	0.0018	0.8248	0.1295	0.0125	0.3811	-

**F value is significant in 1% level, *The F value at 5%. The same letter is not significantly different at further test DMRT level of 5%. DMRT: Duncan multiple test rate

high difference plantlets were not significantly different; of the difference between the numbers of leaves was significantly different. However, for variable number of roots after 8 weeks, there was no increase in the number of roots for ANOVA. The data of the number of roots is still the same as the previous week. Results DMRT 5% of media influence plantlets enlargement treatment were significant to the average difference in height of plantlets (4 weeks after treatment) and the average difference in number of leaves (8 weeks after treatment) for media B.

Induction of SEs of *C. nobilis* Variety SoE

Direct SEs induced from mature seed of *C. nobilis* var. SoE (Tangerine SoE) from NTT. In mature seed consists of two cotyledons, which have the somatic tissue, which called nuclear tissue (Kepiro and Roose, 2007). Research of El-Sawy *et al.* (2006) used ovule explant from immature seeds of ten kinds citrus. Gholami *et al*. (2013) using immature seeds explant of *C. limon* (L.) produce SE in media with composition almost similar, but in MS medium added BA with a concentration of 3 mg/L, sucrose 50 g/L and malt extract 500 mg/L. In this research, we have been using BAP (3 mg/L), sucrose (30 g/L), without malt extract, but vitamin in MS medium we replaced with MW vitamin.

In this study, we showed that the induction of forming SE of this citrus Tangerine SoE is enough using BAP 3 mg/L, without the addition of 2.4-D. Therefore, while 2.4-D added to MS modified + BAP 3 mg/L medium will only hamper forming SE, which desired.

Lee *et al*. (2011) investigated the effect of auxin, in particular for 2.4-D, on the induction frequency of embryogenic callus and SEs from root culture of *Chelidonium majus* after 7 weeks in culture. They have found that 2.4-D concentrations 0.1, 0.5, and 4.0 mg/L were not produced embryogenic callus and embryo somatic, but in level concentrations of 2.4-D 1.0 and 2.0 mg/L, could produce embryogenic callus 15 and 11%, respectively, number of SE per explant very significant 4.9 and 4.5, respectively. Growth regulator such as 2.4-D and BAP often used in general. Different combination of 2.4-D and BAP may show varying results.

Maturation and Germination of SEs of *C. nobilis* SoE

In an investigation by Gholami *et al.* (2013), maturation medium still used the same media to induction forming SEs. In our investigation, ABA used in maturation process to stop the formation of embryogenic callus and stimulate formation embryos from embryogenic callus. Data Table 3 showed that diameter of embryogenic callus was not increased with increasing concentrations of ABA in the medium. After maturation process, the embryos have the potential ability to establish their own hormones to the success of the function during the next phase of development and change (Thiruvengadam *et al.*, 2013), so that it can be said that the addition of GA_3 with a concentration of 2.5 mg/L can support the cotyledon stage embryo germination to form plantlets. After SE formed, then will take happen growth and germination SE, and finally will form a complete plant.

Enlargement of Plantlets

The average plant height at 4 weeks after treatment significantly influenced by media treatment B and C (1/2 and ¼ media concentration). However, the highest average height plantlets (0.1760 cm) due to the influence of the media treatment B. The average number of leaves at week 8 after treatment most widely produced in the media treatment of C (0.83 leaves), but not significantly different from the media treatment of A (0.77 leaves). So that to say that enlargement plantlets using MS modified medium in half-strength concentration. This results the same as the results of research by Park *et al.* (2010) where after germination of SEs, regenerated plantlets recovered on medium containing half-strength MS salts and vitamins without PGR.

In this research, plantlets used as a material in the experiment of enlargement of Tangerine SoE plantlets. In contrast to research conducted by Thirunvengadam *et al*. (2013), use of mature SEs is then produced shoots and roots of poles, hypocotyl elongation improvement and development of the plantlets at a concentration of 1/10 MS medium containing 2% sucrose, 0.5 µM ABA, and 0.2% gelrite within 14 days. MS medium with full concentration containing 3% sucrose and 0.5 µM ABA

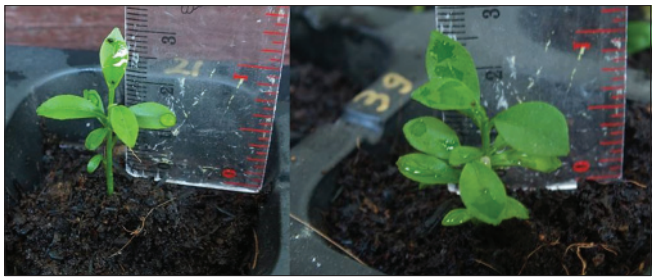

Figure 2: Tangerine SoE plants somatic embryogenesis results have acclimatized

greatly reduced germination and callus embryo. 1/10 MS medium with 2% sucrose and 0.5 µM ABA greatly improve embryo germination and effectively encourage the development of plantlets from SEs, the average frequency or embryo germination into becoming small plants (15%).

Plantlets Acclimatization

A mixture of soil, rice husk, and coconut coir in the ratio 1:1:1 is a source of nutrients for plants and can save the water for a long time. The medium that doused with water to field capacity should not water during 1-2 weeks. Watering can often lead to root plantlets are still learning to look into rotten foodstuffs. Plantlets closed with plastic lid to increase air humidity and placed under the auspices shade to reduce the intensity of incoming light by 40% so that the incoming solar light does not feel any heat. Observation number of plantlets that lives up to 8 weeks after transplanting, there are two plantlets which are of 10 plantlets were acclimatized (Figure 2).

Thiruvengadam *et al.* (2013) move the plantlets into plastic cups containing a mixture of soil, perlite, and vermiculite (3:1:1) yielding 80% of plantlets live and grow up in a greenhouse. The total time of 2 months needed for the production of plants that are ready to transplanting into the pots.

CONCLUSION

Direct SEs have induced from the seeds of a ripe fruit (mature seeds) of *C. nobilis* variety SoE from NTT (Tangerine SoE). Media treatment of induction of SEs with a single BAP 3 mg/L to produce more SEs than the media combination treatment of BAP (3 mg/L) – 2.4-D (0.1, 0.3, and 0.5 mg/L). The concentration of 2.4-D are increasing causing the number of SEs produced diminishing. MS modified media supplemented ABA-PGR concentrations ranging from 0.5 to 3.5 mg/L has not been able to increase the initiation of the initial phase of the formation of globular SEs from embryogenic callus. MS modified medium supplemented with concentrations ranging 0.5-3.5 mg/L media MS modified, can increase the number of plantlets normal at week 8 after treatment. MS modified medium for enlargement plantlets with half-strength concentration may increase the height of plantlets at first 4 weeks and number of leaves at 8 weeks after treatment.

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