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ORIGINAL ARTICLE

Optimization of Synthetic Seed production in MM.106 apple Rootstocks from Somatic Embryos and Axillary buds

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ABSTRACT
Several proliferating programmes have been used for producing apple rootstocks; MM106 is one of rootstocks with many attributes like good induction of cropping, resistance to woolly apple aphid and intermediate vigor. In present study, We used a procedure for micropropagation of apple cv. MM106, treatment of shoots in MS medium culture containing BA (2 mg/l) and IBA (0.1 mg/l) also significantly hastened the regeneration process and increased the number of shoots. In the first subculture, shoots in culture media containing BA were longer and had smaller leaves with thick petioles. In addition, the elongation of stem and branching of MM106 rootstocks were decreased during subcultures. Embryogenic and non-embryogenic calli were produced on MS medium with different concentration hormones. The largest size of non-embryonic calli was observed in T3 and K2 treatments. non-embryogenic calli of MM106 were friable, white, and translucent. The earliest visible sign of somatic embryogenesis was noticeable within 5–10 days of culture. Embryonic calli were produced in T5, T13, T15 and K2 treatments. MM106 embryogenic callus was firm, yellow, and opaque. Embryo production was determined by counting embryos at the heart and torpedo stages of development at 21 days. Somatic embryos and apical and axillary buds of rootstock were encapsulated by mixing with sterilized 3% (w/v) Sodium alginate solution containing 3% sucrose.. Germination frequency of synthetic seeds at 4 ºC was checked up to 10 weeks, Germination generally did not occur during 10 weeks.
***KEYWORD:** Synthetic seed, rootstock, embryonic calli, non-embryonic calli*

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INTRODUCTION

Apple (*Malus× domestica* Borkh.) is one of the most widespread and popular fruit trees in the world [1]. About 59 species and 7500 cultivars were identified in all over the world.
Considering world fruit production, apple (Malus spp.) is the third most important fruit crop (64.3 million t/year) after banana (81.3 million t/year) and grape (66.3 million t/year) [2]. Breeding of apples by conventional hybridization requires many years because of their long juvenile period, a high level of self-incompatibility, and the concomitant highly heterozygous nature of the genome. The use of biotechnological methods in apple breeding offers a way to bypass the disadvantages of sexual hybridization [3]. However, for this to occur, regeneration of adventitious shoots and micropropagation are necessary for breeding plants via nonsexual methods (somaclonal variants, Agrobacterium-mediated transformation, *in vitro* mutagenesis and artificial seed production).
Several breeding programmes for apple rootstocks have produced new apple rootstock clones. Among them, MM106 has many attributes, i.e. good induction of cropping, resistance to woolly apple aphid and intermediate vigor. In apple, *in vitro* culture is considered to be the most effective method for mass clonal multiplication. True to type clonal fidelity is one of the most important pre-requisite in the synthetic seed production of apple rootstocks.
Somatic embryogenesis has been used as a good alternative to the propagation of selected trees (Bueno [4], Manzanera [5]). A bottle-neck of somatic embryogenesis is the late maturation, acclimation and establishment phase. Maturation has been hampered in many woody species by precocious conversion, spontaneous repetitive embryogenesis, embryo dormancy, and immaturity problems.

Somatic embryogenesis is a powerful tool for the improvement of trees and is considered to be the *in vitro* plant regeneration system of choice in woody plants [6]. In fact, most reports on somatic embryogenesis in woody species describe "embryo-cloning" in which the zygotic embryo is induced to replicate itself indefinitely, and the material being propagated is of unproven genetic value (Merkle [7]). The induction of somatic embryos directly from mature tree tissues or at least from non-seed tissues such as leaf parts or shoot segments is an important objective to be achieved in tree species given that the induction of somatic embryos appears to be associated with the cytological and genetic stability of the regenerated plantlets [8]. Efficient systems based on direct somatic embryogenesis have been developed for numerous species of forest trees [9], [7].

Exogenously supplied growth regulators are essential to the process of somatic embryogenesis [10]. In general, the presence of auxins or substances with auxin activity is necessary for the induction and proliferation of cells that later differentiate into somatic embryos [11], Michalczuk [12]. Although auxins are the best-studied inducers for obtaining embryogenic cells, they are certainly not unique in the ability to mediate the transition from somatic cells to embryogenic cells [13]. Cytokinins has been shown to provide sufficient stimulus for inducing somatic embryogenesis in a variety of plant species, including peanut, tobacco and geranium [14-16] also, the combination of auxin and cytokinin requirements of somatic embryogenesis.

Synthetic seed production is an applied technology which capitalizes on the capacity for plant multiplication via somatic embryogenesis. Artificially encapsulated somatic embryos can be sown under *in vitro* or ex vitro conditions, producing uniform clones [17).

A well-tuned somatic embryogenesis system is also required for synthetic seed production. Synthetic, artificial or somatic seeds are analogous to the true or botanical seed, and consist of a somatic embryo surrounded by one or more artificial layers forming a capsule (Cangahuala-Inocente [18]. Studies on synthetic seed production using somatic embryos have been reported in a few forest species, such as *Paulownia elongata* [19], *Eucalyptus citriodora (*Muralidharan [20] and *Chamaecyparis pisifera* [21].

Synthetic seed technology is a good substitute to traditional seeds and micropropagation systems as the establishment of germplasm repositories of traditionally micropropagated plants for further study is difficult; due to limited space; huge amount of money is required for their maintenance. Moreover, exchange of stock cultures between laboratories is also problematic in consideration with temperature fluctuations and danger of infestation with microorganisms. Synthetic seeds provide an alternative dependable way for mass scale production, efficient delivery of cloned plantlets and also to meet the international quarantine requirements. Exchange of axenic plant material between laboratories and successful plant regeneration from synthetic seeds has been reported in several plant species (Standardi [22], Maruyama [21]).

In addition, Mathur *et al*. (1989) reported that the use of this technology economized upon the medium, space and time requirements (Mathur [23]). Successful cases of synthetic seeds production and plantlet regeneration have been reported for cereals, vegetables, fruits, ornamentals, aromatic grass and conifers (Fowke [24], Piccioni [25], Castillo [26], Ganapathi [27], Brischia [28], Hao [29], Latif [30]). However, in most cases somatic embryos were used in the encapsulation process. Some authors described the encapsulation of vegetative propagules such as axillary buds or shoot tips (Mathur [23], Ganapathi [27], Latif [30], Sharma [31], Piccioni [25], Standardi [32], Pattnaik [33], Lata [34]), which could be used for mass clonal propagation as well as in long term conservation of germplasm.

MATERIALS AND METHODS

Micropropagation: The MM106 apple rootstock was used in this study. Actively growing shoots of the year were collected in spring from plants pots in greenhouse. One centimeter of internode sample cut and cultured after sterilization. For sterilization, single nodes were taken MM106 rootstock and then ddH2O, NaOCl based on present protocols was used. The MS medium [35] containing 30 gl^{-1} sucrose, 7.0 gl^{-1} agar was used for shooting and branching explants production. The MS media used with 2 mgl^{-1} BA (Benzyl Adenine) and 0.1 mgl^{-1} IBA (Indol Butyric Acid) [36]. The medium was sterilized by autoclaving for 20 min at 121°C. All media containing adjusted pH to 5.8 before autoclaving. Five to six samples were placed into glass jars and maintained at 25±2°C under a 16/8 h light photoperiodic under a light intensity (50 µmol m^{-2} s^{-1} photon flux density) in a germinator. After 30 days, all of samples transferred to fresh medium and processed till third subculture for detection of morphological variations. Morphologic characteristics such as length of shoots in mm, number of branch and number of leaves were evaluated in three subcultures for MM106 rootstock. The experiments were repeated for 3 times for each treatment used and morphological data were analyzed by analysis of variance test (ANOVA) followed by least significant difference test (LSD).

Somatic embryo induction from buds

Culture media

Non-embryonic and embryogenic calli of MM106 were obtained from proliferated buds. The basal medium MS, 30 g/l sucrose and 0.6% agar. Callus proliferation media and embryo production media contained Indol Butyric Acid (0, 1, 2, 3, 4, 5 mg/l) (IBA) and Benzyl Adenine (0.5, 1, 2, 2.5 mg/l) (BA) and Kinetin (0.5 , 1 mg/l) (KIN) and Naphtol Acetic acid (0, 1, 2, 3) (NAA) (Table 2). Embryogenic callus fragments (calli) of 4-week-old cultures were used in the experiments. Five or six calli were cultured per Petri dish, and these were sealed with Parafilm. The cultures were maintained in a growth chamber at 25 ± 2°C under cool-white fluorescent light (50 umol m^{-2} s^{-1} photon flux density) and 16 h photoperiod.

At the end of a monthly proliferation subculture, the same proliferation and calli vessels were transferred to a cold chamber for synthetic seed production.

The experiments were repeated for 3 times for each treatment used and size of callus data were analyzed by analysis of variance test (ANOVA) followed by least significant difference test (LSD).

Encapsulation of buds and somatic embryos:

Somatic embryos and different parts of randomly chosen proliferated shoots from the previous phase, containing apical and axillary buds, were used to obtain 4-mm-long single-node cuttings for somatic embryogenesis and synthetic seed production, which will be referred to as "buds" (Adriani [37]).

Somatic embryos and buds of rootstock were encapsulated by mixing with 3% (w/v) sterilized Sodium alginate solution containing 3% sucrose. Alginate droplets dropped into 75mM Calcium chloride solution under continuous stirring were kept for 30 min., for hardening the coating of Calcium alginate and formed around the embryos. The synthetic encapsulated beads were washed with 5mM CaCl2 solution for one hour and collected by filtration.

Germination of artificial seeds into plantlets:

Encapsulated somatic embryos and buds were germinated on MS basal medium containing 3% sucrose and 0.3% agar and incubated at 25ºC with a 16 h., photoperiod for germination.

RESULTS AND DISCUSSION

The statistical analysis (ANOVA) of all data revealed that under the same conditions for growth regulators affected shoot production, elongation, number of leaves and size of callus significantly ($P < 0.05$). We used a procedure for micropropagation of apple cv. MM106 based on our previous work [3].

Treatment of shoots in MS medium culture containing BA (2 mg/l) and IBA (0.1 mg/l) also significantly hastened the regeneration process and increased the number of shoots. Morphogenesis *in vitro* can be manipulated by controlling *in vitro* conditions such as light, temperature, vessel humidity and osmotic potential via mineral nutrients, carbohydrates and plant growth regulator (cytokinins and Auxins) content of the medium. These conditions interact with intrinsic factors of explants [38,39].

In the first subculture, shoots from media culture containing BA was more longer and had smaller leaves with thick petioles, Mean length of stem and number of branch were observed 2.14 ± 0.4 (cm) and 2.41 ± 0.3, respectively (Table 1).

The elongation of stem and branching of MM106 rootstocks were decreased during subcultures, in the third subculture, mean of length stem was 0.96 cm and number of branch was 0.94, also number of leaves (14.86) were decreased (Fig. 1).

The LSD analysis showed, length of stem and number of leaves morphological characters were significant different ($P <0.05$), while number of stems were not observed significantly (Table 2). For example, length of stem character was significantly different in B2 treatment and B1 treatment ($P< 0.05$) and number of leaves character showed difference significantly in B2 , B1 and B3 treatments ($P< 0.05$) (Table 2).

Since then, cytokinins have been shown to regulate a variety of biological activities in whole plants and in tissue cultures. They promote outgrowth of axillary buds, stimulate leaf expansion and suppress leaf senescence in whole plants [40]. BA stimulates shoot multiplication in a concentration range of 2.2 up to 4.4, while boosting BA decreases multiplication rate [41].

BA is the most frequently used cytokinin in apple regeneration and it was compared in several studies. Theiler-Hedtrich [42] observed that M9, Golden Delicious and Florina regenerated better on media with TDZ, while Priscilla, M26 and M27 showed best results on media with BA.

BA concentration can have various effects on regeneration of adventitious shoots depending on other factors. These include the use in combination with different levels of IBA. Fasolo *et al*., (1990) showed, when indole-3-butyric acid (IBA) was used as auxin, 'Gala' also needed a low level of BA (4.4 mM) for best regeneration [43].

The optimal plant growth regulator (BA) concentration in the first phase of culture initiation depends on genotype [44], Webster [45], Wang [46], Hofer [47]). For example, BA at 22.0 µM was found optimal to obtain shoot multiplication of 'M26' Welander [48], 'Jork' [49], 'MM106' [50], while the optimal range of BA was from 22.2 to 44.4 µM in 'Florina' [51].

Non-embryogenic Callus and embryogenic callus Induction

Embryogenic and nonembryogenic calli productions on media culture were determined at 4 weeks. The largest size of non-embryonic calli were 7.7 mm in T3 (BA 0.5 mg/l, IBA 2 mg/l) and 5.3 mm in K2 (KIN. 0.5 mg/l, NAA 1 mg/l) treatments. MM106 nonembryogenic calli were friable, white, and translucent (Table 3).

The LSD analysis showed that significantly difference in size of calli in some of treatments ($P<0.05$). For example, size of embryonic calli showed significant difference between T3 and all other treatments studied (expect T5, T15 embryonic calli treatments; $P< 0.05$) (Table 4). Also, size of embryonic calli was significantly different in K2 treatment and rest of treatments (expect K3 and K4 non-embryonic calli treatments; $P< 0.05$) (Table 5).

Callus proliferation was necessary to obtain suitable quantities of embryogenic tissue. Cell proliferation is a result of callus passages (each 2–3 weeks) onto fresh media of identical or amended composition in relation to the composition of initial medium. Increasing the concentration of cytokines in the medium for propagation has a beneficial effect on callogenesis because these regulators stimulate cell division processes. Cytokines such as BA (Benzyl amino purine), KIN (Kinetin), TDZ (Thidiazuron) promote the proliferation of callus and somatic embryo formation in the globular stage. Cytokines stimulate the biosynthesis of nucleic acids, structural proteins and enzymes, inhibit the activity of ribonuclease and protease, and accelerate cell division [52].

Somatic embryo initiation

Not all callus lines were embryogenic and capable of intensive proliferation. Only certain genotypes were characterized by high frequency of embryogenesis.

The earliest visible sign of somatic embryogenesis was noticeable within 5–10 d of culture. Embryonic calli were produced in T5 (BA 0.5 mg/l, IBA 4 mg/l), T13 (BA 2 mg/l, IBA 0 mg/l), T15 (BA 2 mg/l, IBA 2 mg/l) and K2 (KIN 0.5 mg/l, NAA 1 mg/l) treatments. MM106 embryogenic callus were firm, yellow, and opaque (Fig. 3). Embryo production was determined by counting embryos at the heart and torpedo stages of development at 21 days. Somatic embryos at the torpedo stage with 3 to 4 mm in size and translucent appearance, were maintained in 9 cm diameter Petri dishes containing 25 ml basal culture medium.

The origin of plant material, particularly buds, has a significant impact on embryogenic capacity of callus. First somatic embryos are formed in a globular stage. This is the induction of somatic embryos. Also the explant itself, e.g. a leaf and bud, have a particular meaning in the process of embryo induction on a proliferated callus. the younger innermost leaf and bud tissues (constituting the primary explants) produce callus with large quantities of somatic embryos, while further - more external - parts of leaves may produce smaller callus or the embryos are induced directly on this leaf fragment [53].

The somatic embryos were kept in light for one month at 25°C, with four embryos per Petri dish. Then, they were rehydrated individually in 15 cm-long and 2.5 cm diameter test tubes containing 10 ml distilled sterile water for 24 h at 4°C in darkness prior to encapsulation and conversion.

Encapsulation with alginate gel:

The idea of synthetic seeds or artificial seeds was first conceived by Murashige in 1977 [10]. Initially, the development of synthetic seeds had been restricted to encapsulation of somatic embryos and buds in a protective jelly.

The coating protects the explants from mechanical damage during handling and allows germination and conversion to occur without inducing undesirable variations [54]. They behave like true seeds and sprout into seedlings under suitable conditions. Its potential advantages include stabilities during handling, potential for long term storage without losing viability, transportation and planting directly from *in vitro* to field conditions and higher scale at a low cost production [55].

Encapsulated buds excised from in vitro proliferated shoots of MM106 apple rootstock, buds and somatic embryos encapsulation seems to be one of the promising methods for sowing buds and embryos, because encapsulation with the proper materials and structure will not only protect buds and embryos from physical damage or desiccation during the delivery or sowing process in the greenhouse, but also enable easy handling and automation.

Alginate hydrogel was frequently selected as a matrix for synthetic seed because of its features including moderate viscosity and low spin ability of the solution, low toxicity and quick gelation which is an important characteristic for the application of droplet hardening method [56]). Alginate capsules were made quickly by dropping Sodium alginate solution (3%) with somatic embryos and buds into hardening solution, 75mM Calcium chloride (droplets hardening methods). Capsules were hardened for 10 to 30 min., followed by rinsing with auto-dist H2O to remove excess Calcium ions (Fig. 3).

The capsules were weak and shoot tips dried and turned brown, at low concentration (2%) of sodium alginate. However, at higher levels (4%) of sodium alginate, capsules were so hard that they prevented shoot tip proliferation. An optimal concentration of 3% sodium alginate and 100 mM CaNo3.4H2O

solutions gave uniform, firm and identical capsules within our treatments [57]. Soneji et al., (2002) reported that a concentration of 3% sodium alginate was most effective for shoot encapsulation in *Ananas cosmosus* [58].

Germination efficiency of synthetic seeds:

Germination frequency of synthetic seeds stored at 4 ºC was checked up to 10 weeks (Fig. 4). Germination generally did not occur during 10 weeks.

The physical treatment such as cold, heat, osmotic or nutrient stress apparently triggers a process leading to the expression of desiccation tolerance [59, 60]. It can elicit a similar response, presumably because they stimulate the endogenous synthesis of ABA [61].

Sarmah et al., (2010) reported that synthetic seeds stored at 4°C could reduce the germination percentage [62], which was observed in the present study. Higher germination percentage in case of synthetic seeds (without storage) could be due to the matrix, which not only facilitates regular nutrient supply but also protects delicate tissue from any mechanical injury during handling and desiccation. Hardness in capsules causing anaerobic environment inside the capsules may inhibit axillary buds respiration. Hardness or rigidity of the beads mainly depends on the number of Na+ ions exchanged with Ca- ions. At the same time, internal factors related to developmental stage of axillay buds could also be one of the important limiting factors affecting germination [62].

In conclusion, synthetic seed technology would allow production of mass propagation material of MM106 rootstock. This technique has a tremendous potential for scaling up the micropropagation procedure while at the same time economizing upon time, space and cost. Besides, the use of vegetative propagation assures a high degree of genetic uniformity and stability, minimizing the occurrence of somaclonal variations.

Figure 1- a) Shoot length, b) branching, c) leaf morphology in MM106 apple rootstock from MS medium BA (2 mg/l) and IBA (0.1 mg/l)

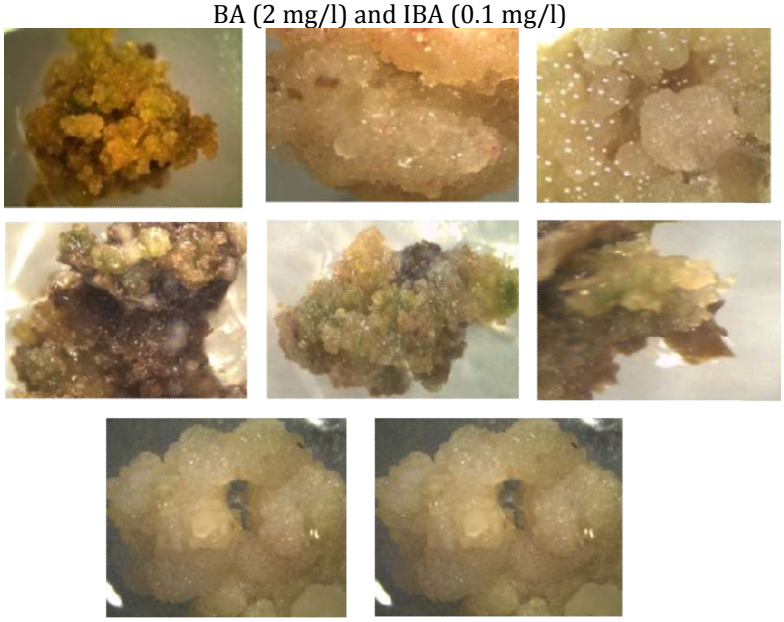

Figure 2- Non-embryonic callus (right) and embryonic callus (left) from A) T5 (BA 0.5 mg/l- IBA 4 mg/l) treatment, B) T13 (BA 2 mg/l- IBA 0 mg/l) treatment and C) T15 (BA 2 mg/l- IBA 2 mg/l) treatment D) Embryonic callus (right) and Somatic embryo (left) from K2 (KIN 0.5 mg/l- NAA 1 mg/l) treatment in MM106 apple rootstock

Figure 3- Synthetic seed of production by buds in MM106 apple rootstock

Table 1- Mean of length stem, number of branch and number of leaves in MM106 apple rootstock during 3 subcultures

No. Subculture	Mean lenght of stem (cm)	Mean number of branch	Mean number of leaves
1	2.14 ± 0.4	2.41 ± 0.3	15.00 ± 2.8
2	1.07 ± 0.4	2.27 ± 1.03	18.68 ± 7.4
3	.96 ± 0.4	.94 ± 0.4	14.86 ± 5.8

Table 2- Representative mean difference test (LSD) for morphological characters among three subcultures (BA and IBA; [36]

(I)Lenght of stem B2	(J) Lenght of stem B3	Mean Difference (I-J) 3.9369*	Significant .043
(I)Lenght of stem B3	(J) Lenght of stem B2	Mean Difference (I-J) -3.9369*	Significant .043
(I)Number of leaves B1	(J) Number of leaves B3	Mean Difference (I-J) .5229*	Significant .001
(I)Number of leaves B2	(J) Number of leaves B3	Mean Difference (I-J) .2729*	Significant .026
(I)Number of leaves B3	(J) Number of leaves B1	Mean Difference (I-J) -.5229*	Significant .001
	B2	-.2729*	.026

* The mean difference is significant at the .05 level.

Table 3- Mean of size non-embryonic calli and embryonic calli in MM106 apple rootstocks from MS media with BA and IBA (Jacoboni, satandardi 1982) and KIN and NAA (Daigny et al., 1998) different concentrations

Treatments	BA (mg/l)	IBA (mg/l)	KIN (mg/l)	NAA (mg/l)	Mean of size calli (mm)
T1	0.5	0	_	_	1.70± 0.44
T2	0.5	1	_	_	2.40 ± 0.63
T3	0.5	2	_	_	7.70± 4.74
T4	0.5	3	_	_	2.40 ± 0.61
T5*	0.5	4	_	_	5.20± 0.55
T6	0.5	5	_	_	3.30± 0.85
T7	1	0	_	_	2.40 ± 0.77
T8	1	1	_	_	2.90 ± 0.75
T9	1	2	_	_	1.70 ± 0.65
T10	1	3	_	_	3.40 ± 0.71
T11	1	4	_	_	2.20 ± 0.85
T12	1	5	_	_	2.30 ± 0.75
T13*	2	0	_	_	2.50 ± 0.79
T14	2	1	_	_	2.70 ± 0.73

T15*	2	2	_	_	5.50 ± 0.56
T16	2	3	_	_	2.50 ± 0.65
T17	2	4	_	_	3.00 ± 0.81
T18	2	5	_	_	4.00 ± 0.81
T19	2.5	0	_	_	2.60 ± 0.84
T20	2.5	1	_	_	5.40 ± 0.54
T21	2.5	2	_	_	5.80 ± 0.57
T22	2.5	3	_	_	5.90 ± 0.62
T23	2.5	4	_	_	6.40 ± 0.67
T24	2.5	5	_	_	5.10 ± 0.50
K1	_	_	0.5	0	4.5 ± 0.71
K2*	_	_	0.5	1	5.3 ± 0.84
K3	_	_	0.5	2	3.3 ± 0.7
K4	_	_	0.5	3	3.8 ± 0.81
K5	_	_	1	0	4.1 ± 0.87
K6	_	_	1	1	4.2 ± 0.86
K7	_	_	1	2	4.6 ± 0.56
K8	_	_	1	3	5.3 ± 0.55

*Embryonic calli

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Table 4. Representative mean difference test (LSD) for size of callus among treatments (BAP and IBA different concentrations; Jacoboni, satandardi 1982), * The mean difference is significant at the .05 level.

(I)size of callus T1	(J)size of callus T3	Mean Difference (I-J) -6.00 *	Sig. .000	(I)size of callus T12	(J)size of allus T22	Mean Difference (I-J) -3.60*	Sig. .032
	T5	-3.50*	.038		T23	-4.10*	.015
	T20	-3.70*	.028	(I)size of callus T13	(J) size of callus T3	-5.20*	Sig. .002
	T21	-4.10*	.015		T21	-3.30*	.050
	T22	-4.20*	.013		T22	-3.40*	.043
	T23	-4.70*	.005		T23	-3.90*	.021
	T24	-3.40*	.043	(I)size of callus T14	(J) size of callus T3	-5.00*	.003
(I)size of callus T2	(J)size of callus T3	-5.30*	Sig. .002		T23	-3.70*	Sig. .028
	T21	-3.40*	.043	(I)size of callus T15	(J) size of callus T1	3.80*	.024
	T22	-3.50*	.038		T9	3.80*	.024
	T23	-4.00*	.018		T11	3.30*	.050
(I)size of callus T3	(J)size of callus T1	6.00*	Sig. .000	(I)size of callus T16	(J) size of callus T3	-5.20*	Sig. .002
	T2	5.30*	.002		T21	-3.30*	.050
	T4	5.30*	.002		T22	-3.40*	.043
	T6	4.40*	.009		T23	-3.90*	.021
	T7	5.30*	.002	(I)size of callus T17	(J) size of callus T3	-4.70*	.005
	T8	4.80*	.005		T23	-3.40*	.043
	T9	6.00*	.000	(I)size of callus T18	(J) size of callus T3	-3.70*	.028
	T10	4.30*	.011	(I)size of callus T19	(J) size of callus T3	-5.10*	.003
	T11	5.50*	.001		T22	-3.30*	.050
	T12	5.40*	.001		T23	-3.80*	.024
	T13	5.20*	.002	(I)size of callus	(J) size of callus T1	3.70*	.028

				T20			
	T14	5.00*	.003		T9	3.70*	.028
	T16	5.20*	.002	(I)size of callus T21	(J) size of callus T1	4.10*	.015
	T17	4.70*	.005		T2	3.40*	.043
	T18	3.70*	.028		T4	3.40*	.043
	T19	5.10*	.003		T7	3.40*	.043
(I)size of callus T4	(J)size of callus T3	-5.30*	Sig. .002		T9	4.10*	Sig. .015
	T21	-3.40*	.043		T11	3.60*	.032
	T22	-3.50*	.038		T12	3.50*	.038
	T23	-4.00*	.018		T13	3.30*	.050
(I)size of callus T5	(J)size of callus T1	3.50*	Sig. .038		T16	3.30*	Sig. .050
	T9	3.50*	.038	(I)size of callus T22	(J) size of callus T1	4.20*	.013
(I)size of callus T6	(J)size of callus T3	-4.40*	Sig. .009		T2	3.50*	Sig. .038
(I)size of callus T7	(J)size of callus T3	-5.30*	Sig. .002		T4	3.50*	Sig. .038
	T21	-3.40*	.043		T7	3.50*	.038
	T22	-3.50*	.038		T9	4.20*	.013
	T23	-4.00*	.018		T11	3.70*	.028
(I)size of callus T8	(J)size of callus T3	-4.80*	Sig. .005		T12	3.60*	Sig. .032
(I)size of callus T9	(J)size of callus T3	-6.00*	Sig. .000		T13	3.40*	Sig. .043
	T5	-3.50*	.038		T16	3.40*	.043
	T15	-3.80*	.024		T19	3.30*	.050
	T20	-3.70*	.028	(I)size of callus T23	(J) size of callus T1	4.70*	.005
	T21	-4.10*	.015		T2	4.00*	.018
	T22	-4.20*	.013		T4	4.00*	.018
	T23	-4.70*	.005		T7	4.00*	.018
	T24	-3.40*	.043		T8	3.50*	.038
(I)size of callus T10	(J)size of callus T3	-4.30*	Sig. .011		T9	4.70*	Sig. .005
(I)size of callus T11	(J)size of callus T3	-5.50*	Sig. .001		T11	4.20*	Sig. .013
	T15	-3.30*	.050		T12	4.10*	.015
	T21	-3.60*	.032		T13	3.90*	.021
	T22	-3.70*	.028		T14	3.70*	.028
	T23	-4.20*	.013		T16	3.90*	.021
(I)size of callus T12	(J)size of callus T3	-5.40*	Sig. .001		T17	3.40*	Sig. .043
	T21	-3.50*	.038		T19	3.80*	.024
	T22	-3.60*	.032	(I)size of callus T24	T1	3.40*	.043

Table 5. Representative mean difference test (LSD) for size of callus among treatments (KIN and NAA different concentrations; Daigny et al., 1998)

(I)size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K1	K3	.8333 *	.001
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K2	K3	.7667 *	.002
	K4	.4667*	.043
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K3	K1	-.8333 *	.001
	K2	-.7667 *	.002
	K5	-.6000 *	.012
	K6	-.5333 *	.023
	K7	-.4667 *	.043
	K8	-.5667 *	.017
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K4	K1	-.5333 *	.023
	K2	-.4667 *	.043
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K5	K3	.6000 *	.012
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K6	K3	.5333 *	.023
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K7	K3	.4667 *	.043
(I)Size of callus	(J)Size of callus	Mean Difference (I-J)	Sig.
K8	K 3	.5667 *	.017

* The mean difference is significant at the .05 level.

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