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Germination and Subsequent Plant Development of *in vitro* Cultured Zygotic Embryos and Embryonic Axes in Comparison to Conventional Seed Propagation of Tea (*Camellia sinensis* L.)

Thayamini H Seran[1*], M T K Gunasekare[2] and K Hirimburegama[3]
([1]*Department of Agronomy, Eastern University, Chenkalady, Sri Lanka;*
[2]*Division of Plant Breeding, Tea Research Institute, Talawakelle, Sri Lanka;*
[3]Department of Plant Sciences, University of Colombo, Colombo, Sri Lanka)

ABSTRACT

The present study was carried out to achieve synchronous germination of plant materials into vigorous plants of tea *(camellia sinensis* L.), under *ex vitro* conditions. Sterilized zygotic embryos and zygotic embryonic axes were cultured on MS basal medium supplemented with 3 mg L^{-1} BAP and 0.5 mg L^{-1} IBA. To serve as controls, seeds were sown in a sand bed.

At the 8th week, *in vitro* plantlets were transferred to ex *vitro* conditions for acclimatization, and seeds showing different germination responses were separately transplanted. Synchronous and significantly high germination (99%) was observed from cultured embryonic axes *in vitro* at the 4th week of culture, but a lower level of germination (12.7%) from seeds that were sown in the sand bed. Further, healthy plantlets regenerated from embryonic axes *in vitro* had erect shoots with short internodes, as well as tap roots with abundant adventitious roots under *ex vitro* conditions, for better adaptation in the field, as compared with seedlings raised by conventional seed propagation.

Key Words: Germination, *in vitro* Culture, Plant Conversion, Seed, Zygotic Embryo, Zygotic Embryonic Axis.

INTRODUCTION

As tea is a heterozygous plant, there is considerable genetic variability in seedling populations (Purseglove, 1984; Anandappa, 1986). The selection method is widely adopted on progeny to identify superior individuals among existing genotypes. Thus, new cultivars have been developed, mostly by the Tea Research Institute, to increase the quality and quantity of tea production in Sri Lanka.

However, the selection of potential cultivars is a long and laborious process (Anandappa, 1986). Flowers are produced in abundance in the tea seed trees but the

percentage of fruit set is generally less than 14% (Harler, 1956; Bezbaruah, 1975; Anandappa *et al.*, 1988). Further, it varies with the clones (Bezbaruah, 1975; Anandappa *et al.*, 1988), and high-yielding clones show very low seed set (Anandappa *et al.*, 1988). Therefore, germination of hybrid seeds plays an important role in developing a relatively large number of seedling progeny during the initial phase of crop improvement, for better selection of superior individuals under nursery conditions.

The tea seed shell is a mechanical barrier to its germination (Visser and Tillekeratne, 1958; Sebastiampillai and Anandappa, 1979; Chaudhury *et al.*, 1990). Cracking or removal of the testa, or soaking seeds in water and then sun-drying them for a few hours, before sowing in a sand bed, accelerated the germination of fresh seeds (Sebastiampillai and Anandappa, 1979; Purseglove, 1984). However, germination of the naked embryos is little affected by pre-soaking (Sebastiampillai and Anandappa, 1979), and the complete removal of the shell requires considerable care and destroys the mechanical protection it gives the seed (Tubbs, 1932).

Owing to the failure of crossed materials to germinate in the nursery, it is necessary to raise hybrid seedlings *in vitro* through the embryo culture technique (Kato, 1989) that may help tea breeders to shorten the breeding cycle of tea (Bezbaruah, 1975).

A very high rate of germination (93-100%) was reported from cultured embryonic axes (Seran *et al.*, 2005). Therefore, it would be beneficial to germinate the excised embryonic axes of hybrid seeds *in vitro*, in plant breeding lines. Roots formed *in vitro* are often non-functional (Debergh and Maene, 1981), but tea seedlings raised in a sand bed have a deep tap-root system. Marimuthu *et al.* (1998) reported that tissue-cultured plants, obtained by micropropagation from cuttings of elite tea cultivars, are more vigorous than vegetatively-propagated plants. Even though several authors have proven the possibility of mass propagation of tea through *in vitro* propagation techniques, work on performance of *in vitro* regenerated plants under *ex vitro* conditions is limited.

There have been no investigations on *in vitro* plant development by zygotic embryos and embryonic axes under *ex vitro* conditions, as compared to seedlings raised in sand beds. Therefore, in this study, the performance of plants, regenerated from embryos and embryonic axes cultured *in vitro*, was examined to assess their germination and subsequent growth under *in vitro* and *ex vitro* conditions, as compared to conventional seed propagation.

MATERIALS AND METHODS

Plant material

Mature fruits of the tea cultivar, TRI 2043, were collected from seed-bearers at the Tea Research Institute, St.Coombs, Talawakelle. After removal of the fruit-coat from harvested fruits, all cracked, infected or damaged seeds were discarded, and seeds of uniform size were tested for viability by the sinker-and-floater method (Barua, 1989).

Zygotic embryos (each consisting of a single cotyledon with embryonic axis), and zygotic embryonic axes (de-cotylated zygotic embryos), were carefully isolated from two-thirds of the mature seed stock. Subsequently, zygotic embryos were surface-sterilized in a 20% solution of Clorox™ (sodium hypochlorite, 5.25% active ingredient) for 30 min, while embryonic axes were surface sterilized in 2% Clorox™ for 10 min. They were then rinsed thoroughly in sterilized distilled water before being cultured on germination medium. The remaining one-third of the seed stock was used as the control.

Culture conditions for seedling and *in vitro* plantlet regeneration

The sterilized embryonic axes and zygotic embryos were placed on MS basal medium, supplemented with 3 mg L^{-1} BAP, 0.5 mg L^{-1} IBA, and 3% (w/v) sucrose, and solidified with 0.8% (w/v) agar (Seran *et al.*, 2005). The seeds used as controls were sown in a sand bed following a standard method. Each embryonic axis was cultured in a culture-vial (a 28 ml McCartney bottle with an aluminum screw cap) containing 8 ml medium, while three of the zygotic embryos were placed together in bottles (of 125 ml capacity) containing 20 ml medium. The convex surface of the abaxial side of the cotyledons was in contact with the culture medium.

All cultures were incubated at 22 ± 2°C under white fluorescent light (16 h photoperiod, 25 μmoles m^{-2} s^{-1}). The orientation of germinated embryonic axes was corrected after four weeks of culture. Two independent experiments were conducted. The frequency of germination in each of the plant materials tested was assessed every week, and the different germination responses were recorded at the 8th week of incubation.

Establishment of seedlings and *in vitro* plantlets under *ex vitro* conditions

At the 8th week of sowing, seeds showing different responses were separated into four groups and planted separately in standard nursery bags (23 cm in height, 13 cm in diameter) containing soil. The plantlets regenerated *in vitro* were planted in small nursery bags (11.5 cm height, 6.5 cm diameter) containing the same soil.

These poly bags were randomly arranged in the nursery bed and covered with a sealed polythene cover, as described by Kathiravetpillai *et al.* (1976). Hardening was done after 8 weeks of transplanting; thereafter, plants were transplanted to standard nursery bags after the formation of 1-2 new leaves.

All agronomic practices were carried out as described by Kathiravetpillai and Kulasegaram (1986).

Observations on plant development were made every week and plant growth (shoot length, number of leaves, etc.) was assessed with the plants available. Damaged or sun-scorched plants at any stage were not taken into account in evaluating growth characters.

STATISTICAL ANALYSIS

Data were analysed using SAS software. All data were first subjected to the Shapiro-Wilk test ($P = 0.05$) for normality, before subjecting them to analysis of variance (ANOVA). Transformation techniques were used for values expressed as percentages, and also for shoot or root lengths. The significant differences between means were estimated using the Duncan's Multiple Range Test at the 5% level of significance.

RESULTS

Germination of plant material under *in vitro* and *ex vitro* conditions

Zygotic embryos and zygotic embryonic axes, cultured on the germination medium, gradually turned green during the first week of incubation. Both zygotic embryos and embryonic axes cultured *in vitro* germinated after a week, whereas seeds sown in the sand bed took three weeks to germinate. Synchronous germination was observed in embryonic axes cultured *in vitro*, in the plant materials tested.

The frequencies of cumulative germination of zygotic embryos and embryonic axes cultured *in vitro*, and seeds sown in the sand bed, are shown in Table 1. The presence of cotyledons in zygotic embryos showed different germination responses under *in vitro* conditions (Fig. 1 A, B). The cotyledonary petioles of some of the zygotic embryos (6.7%) became swollen, and thereafter embryoids appeared on the swollen surface over 6 - 8 weeks.

Growth responses of the seeds sown in the sand bed, and the zygotic embryos cultured under *in vitro* conditions, were found to be similar (Table 2). The plant materials exhibiting various germination responses after being in the sand bed and *in vitro* conditions for 8 weeks were categorized into four groups:

1) seedling or plantlet formation;
2) root development only;
3) non-germinated seeds;
4) shoot development only at the 8th week after the sowing of seeds.

Seeds in group 3) germinated after 8 - 10 weeks of sowing in the sand bed.

Plant growth and morphology under *ex vitro* conditions

At the 8th week of culture, *in vitro* plantlets showed better shoot growth than root growth, when compared to seedlings grown in the sand bed; the latter showed better root growth than shoot growth (Table 3, Figure 1C).

The mean shoot length of seedlings raised in the sand bed varied among groups 1, 2 and 3 (Table 4), and also the differences in mean shoot length of plants obtained under both *in vitro* and *ex vitro* conditions were highly significant at each period. However, no significant differences in mean number of nodes, or mean number of leaves, were obtained in the different plant categories tested (Table 5). The stem girth of seedlings was thicker than that of *in vitro* plantlets.

The shoot length of plants regenerated from embryonic axes *in vitro* increased continuously, and attained a mean shoot length of 20 cm at 12 months (52 weeks), and 30 cm at 15 months, after culture. These plants had erect shoots with short internodes and tap roots with numerous adventitious roots (Figures 1 D, E and F).

DISCUSSION

The results of this study were that the rates of germination varied significantly among plant materials at each period. In agreement with previous results (Seran *et al.*, 2005), embryonic axes cultured under *in vitro* conditions exhibited early germination, and attained a very high rate of germination (99.0%, Table 1) after four weeks of initiation of the culture.

Eight weeks after sowing, only 26.7% of the seeds developed into seedlings, whereas in 31.0% of the seeds only root development was observed with no emergence of the plumules (Table 2). The results confirm that the testa in tea seeds is a mechanical barrier to germination (Visser and Tillekeratne, 1958; Sebastiampillai and Anandappa, 1979; Chaudhury *et al.*, 1990).

In the present study, the rates of cumulative germination of seeds at the 12th and 16th weeks after sowing were 70% and 85%, respectively, when emergence of the plumule was considered as the indicator of germination. In contrast, Sebastiampillai and Anandappa (1979) reported that 90% of seeds sown in a sand bed germinated at the end of eight weeks, when the emergence of the radicle was taken as the indicator of germination.

No distinct difference in the germination response was observed between seedlings raised by conventional methods and plantlets developed from zygotic embryos *in vitro*. These results show that the seed coat and the seed cotyledon are critical factors that contribute to non-synchronous and delayed germination. However, food reserves in the cotyledons promote good embryonic growth (Anandappa, 1986). Therefore, mean shoot and root growth of seedlings raised in the sand bed, and plantlets established from zygotic embryos *in vitro*, were higher than those of plantlets developed *in vitro* from cultured embryonic axes, at the 8th week. Further, root growth of seedlings propagated by conventional methods was higher than their shoot growth.

According to Fahn (1967), during germination, the radicle generally emerges after rupturing of the testa at the micropylar end, and then penetrates into the soil. After this, further rupturing of the testa takes place. It is evident that there is a faster growth of roots in the seedlings (the controls).

The ratio of shoot: root growth was higher in plantlets obtained from embryonic axes *in vitro* than in seedlings raised in the sand bed, at the 8th week. Etienne *et al.* (2002) reported that shoot emergence from the somatic embryos of coffee was more efficient *in vitro* than under *ex vitro* conditions, and that plantlets regenerated *in vitro* had more leaves, although their rooting frequency was low. Roots formed *in vitro* are often non-functional (Debergh and Maene, 1981).

The survival rates of *in vitro* plantlets established from embryonic axes and from zygotic embryos were 50% and 100%, respectively, at the 8th week after transplanting into the soil. In a previous, unpublished study, a higher rate (about 96%) of survival was observed at the 8th week after transplanting of plantlets developed *in vitro* from embryonic axes, and grown in a potting soil mixture (soil: coconut coir dust, 1:1 v/v). Scorching and death of seedlings raised in the sand bed was observed in the nursery after transplanting. This finding is supported by the work of Nanda and Rout (2003), who reported that propagation through seeds of *Acacia arabica*, as with most leguminous trees, is unreliable owing to poor germination and death of young seedlings under natural conditions.

Ex vitro shoot growth of plantlets regenerated *in vitro* may be affected during the 8 - 27 weeks after culture initiation, owing to the hardening practice. Probability values

of the F-ratio at 52 and 47 weeks of growth are 0.01 and 0.001, respectively, but very low (< 0.001) at 8, 27 and 37 weeks. This indicates that variation in shoot growth, among different plant categories, decreases gradually with plant development.

Further, there was no difference in shoot length between the plants established from *in vitro* plantlets and from seedling group 3 at 52 weeks. The shoot lengths of 52-week-old seedlings of group 1 (seeds developed into seedlings at 8th week of sowing), and group 3 (seeds categorized as non-germinated material), were 43.9 cm and 29.4 cm, respectively (Table 4). This indicates that shoot growth of young seedlings in a population is not uniform owing to non- synchronous germination of seed stock sown in the sand bed. This may affect the selection process at the initial phase of plant development in the nursery, when developing a new elite clone.

Plants obtained from embryonic axes cultured *in vitro* had a mean shoot length of 20.0 cm, and a mean number of 12 nodes, at 52 weeks (12 months) after culture. The overall mean shoot length of seedling groups 1 - 3 was 37.1 cm, and this seedling population had an overall mean number of 14 nodes at 12 months after sowing. These results clearly indicate that plants, regenerated from embryonic axes *in vitro*, have shorter internodes than that of *ex vitro* seedlings.

Generally tea seedlings are field-planted when they are about 20 cm in height, and before their tap roots reach the bottom of the polythene sleeves (Anandappa, 1986). Conventional seed propagation takes about 8 - 10 months to develop young seedlings (30 – 50 cm shoot height) that are ready for field-planting. As a preliminary investigation, a few plants out of 15-month-old plants regenerated from embryonic axes and cultured *in vitro* were planted in the field. Shoots of these plants were straight and showed no signs of wilting after planting. Stem girths (base to top portion), that were more or less similar, were observed in plants regenerated from embryonic axes *in vitro*, unlike in seedlings established in the sand bed. Furthermore, plants obtained from embryonic axes *in vitro* produced more fibrous roots from the tap root, under *ex vitro* conditions, in comparison to seedlings obtained through seed propagation.

The present study indicates that *in vitro* propagation using embryonic axes of hybrid tea seeds would be useful in achieving synchronous germination, giving rise to vigorous, comparatively disease-free plants which have abundant fibrous roots for better establishment in the field.

Future studies will be focused on comparing the field performance of plants, regenerated from embryonic axes cultured *in vitro*, and conventional seedlings.

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REFERENCES

Anandappa T I 1986 Planting material. In: *Handbook on Tea.* (eds. P Sivapalan, S Kulasegaram, A Kathiravetpillai), p. 6 – 19. The Tea Research Institute of Sri Lanka.

Anandappa T I, Nanayakkara R and Solomon H R 1988 Seed setting abilities of some Sri Lankan tea clones and their implications for tea breeding. In: *Proceedings of the Regional Tea (Scientific) Conference.* (eds. P Sivapalan, A Kathiravetpillai), p. 73-87.

The Tea Research Institute, Sri Lanka.

Barua D N 1989 Propagation technique. In: *Science and Practice in Tea Culture* (ed. D N Barua), p. 226. Tea Research Association, Calcutta, India.

Bezbaruah H P 1975 Pollination in tea (*Camellia sinensis* L. O. Kuntze) in North-East India. Two and a Bud 22, 61- 63.

Chaudhury R, Lakhanpaul S, Chandel K P S and Singh I D 1990 Germination and desiccation tolerance of tea (*Camellia sinensis* L. O. Kuntze) seeds and feasibility of cryopreservation. Sri Lanka J. Tea Sci 59, 89 - 94.

Debergh P C and Maene L J 1981 A scheme for commercial propagation of ornamental plants by tissue culture. Sci Hort 14, 335 - 345.

Etienne B D, Bertrand B, Vasquez N and Etienne H 2002 Comparison of somatic embryogenesis derived coffee (*Coffea arabica* L.) plantlets mineral and water characteristics. Ann Bot 90, 77 - 85.

Fahn A 1967 The Seed. In: *Plant Anatomy* (ed. A Fahan), p. 466-479. Pergamon Press Ltd, Oxford.

Harler C R 1956 *The Culture and Marketing of Tea*, p. 14-15. Oxford University Press, London.

Kathiravetpillai A, Kulasegaram S, Senanayake Y D A and Gunasena H P M 1976 Growth of single and multinodal cuttings of tea (*Camellia sinensis* L. O. Kuntze) under polythene. Cey. J. Nat Agric Soc 13, 1 - 12.

Kathiravetpillai A and Kulasegaram S 1986 Nursery and nursery practices. In: *Handbook on Tea*. (eds. P Sivapalan, S Kulasegaram, A Kathiravetpillai), p. 20 - 35.

Kato M 1989 *Camellia sinensis* L. (Tea): *In vitro* regeneration. In: *Biotechnology in Agriculture and Forestry* (ed. YPS Bajaj), Vol. 7, *Medicinal and Aromatic Plants* II, p. 82 - 98. Springer-Verlag, Berlin, Heidelberg.

Marimuthu S, Balasubramanian S, Kumar R R and Muraleedharan N 1998 Biotechnological approaches in tea research: recent attempts and progress. Ind. Bulletin of UPASI Tea Sci Dept 52, 27 - 33.

Nanda R M and Rout G R 2003 *In vitro* somatic embryogenesis and plant regeneration in *Acacia arabica*.. Plant Cell Tissue Organ Cult 73, 131 - 135.

Purseglove J W 1984 *Tropical crops: Dicotyledons*, p. 599 – 612. The English Language Book Society and Longmam, Green and Co., London.

Sebastiampillai A R and Anandappa T I 1979 The influence of moisture and temperature on the germinability and longevity of tea (*Camellia sinensis* L.) seeds. Tea Q. 48, 8 - 20.

Seran T H, Hirimburegama K and Gunasekare M T K 2005 Encapsulation of embryonic axes of *Camellia sinensis* (L.) O. Kuntze (tea) and subsequent *in vitro* germination. J. Hort Sci Biotech 80,154-158.

Tubbs F R 1932 The germination of tea seed. Tea Q. V, 66 - 69.

Visser T and Tillekeratne L M de W 1958 Observations on the germination and storage of tea pollen and seed. Tea Q. 29, 30 - 35.

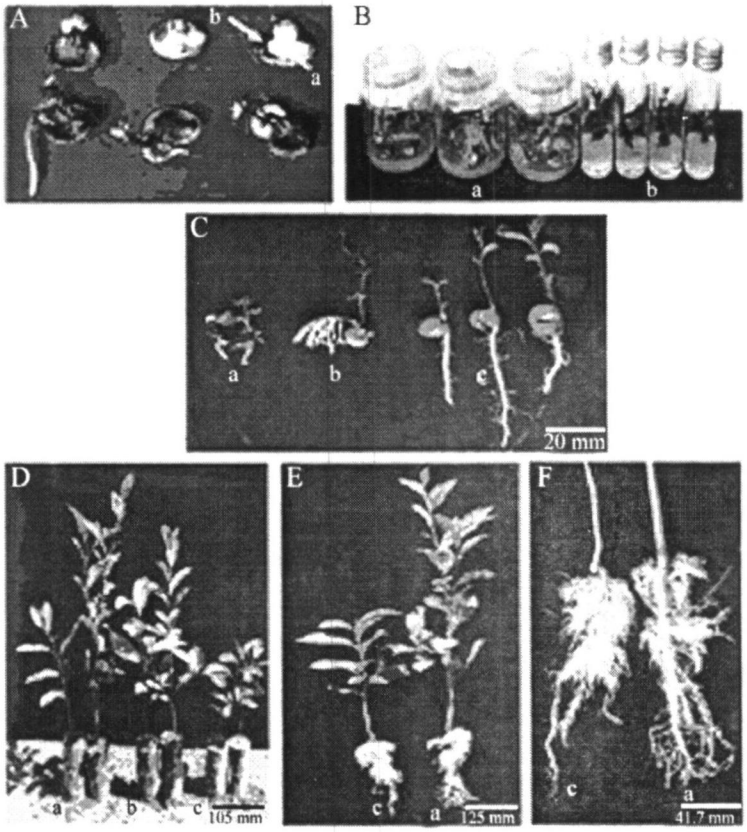

Figure 1

Fig. 1. Plant development from tea seed-derived materials.

A. Germination responses of zygotic embryos *in vitro* at 4th week of culture (a - swelling of tissue; b - cotyledon).

B. Growth responses of zygotic embryos *in vitro* in culture bottles (a) and zygotic embryonic axes in vials (b) at 8th week.

C. Plantlets regenerated from zygotic embryonic axes (a) and zygotic embryos (b) cultured under *in vitro* conditions, and seedlings (c) raised in a sand bed.

D-F. Shoot and root growth of plants established from seeds sown in a sand bed (a), zygotic embryos *in vitro* (b) and zygotic embryonic axes *in vitro* (c) after 15 months of culture.

Table 1. The percentage of cumulative germination of tea plant materials.

Plant material	Cumulative germination %	
	at 4th week	at 8th week
Seeds sown in sand bed	12.7c	26.7c
Zygotic embryos cultured *in vitro*	45.7b	50.7b
Zygotic embryonic axes cultured *in vitro*	99.0a	99.0a
F - test	***	***

The percentages were calculated from 300 samples per treatment.

Means followed by the same letter in each column are not significantly different, according to Duncan's Multiple Range Test at the 5% significance level.

The emergence of plumules in the tested material was considered as the indicator of germination.

F - test: *** : $P = 0.001$.

Table 2. Germination response of tea plant materials after 8 weeks of culture initiation.

Types of germination	Germination response %			
	Seeds in sand bed	Embryos *in vitro*	Embryonic axes *in vitro*	F-test
Seedling/Plantlet formation	26.7b	50.7a	64.7a	**
Shoot development only	0.0	0.0	34.3	-
Root development only	31.0a	24.3a	0.0b	***
Non-germinated material	42.3a	24.0b	1.0c	***

Data based on two independent experiments, each with 150 samples.

Means followed by the same letter in each row are not significantly different, according to Duncan's Multiple Range Test at the 5% significance level.

F - test: ** : $P = 0.01$; *** : $P = 0.001$

Table 3. Growth of plantlets and seedlings under *in vitro* and nursery conditions at the 8th week.

Plant category	Growth parameters		
	Shoot length (cm)	Root length (cm)	Numbers of leaves
Seedlings	3.87 ± 0.21b	8.00 ± 0.04a	3.56 ± 0.17
Plantlets A	5.02 ± 0.34a	5.77 ± 0.70b	2.89 ± 0.26
Plantlets B	2.30 ± 0.27c	1.99 ± 0.22c	3.22 ± 0.34
F - test	***	***	-

Values represent means ± standard errors of two independent experiments.

Means followed by the same letter in each column are not significantly different, according to Duncan's Multiple Range Test at the 5% significance level.

Plantlets A and B refer to those obtained from zygotic embryos and embryonic axes, respectively, cultured *in vitro*.

F - test: *** : $P < 0.001$

Table 4. Mean shoot growth of plants obtained under *in vitro* and nursery conditions at different periods after culture initiation.

Plant category	Shoot growth (cm) at different periods				
	17 weeks	27 weeks	37 weeks	47 weeks	52 weeks
Seedling group 1	08.91a	19.47a	27.84a	34.69a	43.94a
Seedling group 2	07.78ab	18.33a	27.80a	32.75a	37.83ab
Seedling group 3	05.32b	13.70 b	20.60ab	24.90ab	29.40bc
Plantlets A	08.56a	12.78b	18.00b	22.86bc	31.86abc
Plantlets B	02.73c	04.45c	08.03c	14.50c	20.00c

Values are means of two independent experiments.

Means followed by the same letter are not significantly different, according to Duncan's Multiple Range Test at the 5% significance level.

Seedling groups 1, 2 and 3 refer to those obtained from seeds sown in sand bed that showed seedling formation (group 1), root development only (group 2), and non-germinated material (group 3), at the time of transplanting (at 8th week after sowing of seeds).

Plantlets A and B refer to those developed from zygotic embryos and embryonic axes, respectively, under *in vitro* conditions.

Table 5. The mean number of leaves and number of nodes in plants at 52nd week (12th month) of culture initiation.

Plant category	Number of leaves per plant	Number of nodes per plant
Seedling group 1	10.06 ± 0.59	14.75 ± 0.64
Seedling group 2	9.69 ± 1.05	15.08 ± 0.88
Seedling group 3	8.80 ± 1.32	12.40 ± 1.08
Plantlets A	10.57 ± 0.65	14.43 ± 0.75
Plantlets B	7.25 ± 0.75	12.00 ± 1.47
F- test	ns	ns

Values are means ± standard errors of two independent experiments.

Seedling groups 1, 2 and 3 refer to those obtained from seeds sown in sand bed that showed seedling formation (group 1), root development only (group 2), and non-germinated material (group 3), at the time of transplanting (at 8th week after sowing of seeds).

Plantlets A and B refer to those developed from zygotic embryos and embryonic axes, respectively, under *in vitro* conditions.

F - test: ns - not significant