**PLANT REGENERATION FROM CALLUS CULTURES IN ENDANGERED ORCHID *Bulbophyllum auricomum* Lindl.**

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**ABSTRACT**

Callus cultures of *Bulbophyllum auricomum* were successfully established from longitudinally bisected protocorm-like-bodies (PLB). Calli were induced from PLB within one month of culture. Morphologically, two types of calli (yellowish friable and yellowish green friable) were obtained from explants when cultured on basal medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) alone or combined with 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ). The frequency of callus induction varied with concentrations and combinations of exogenously applied plant growth regulators (PGRs); and hundred percent callus induction was achieved on MS basal medium supplemented with 1.0 mgl-1 TDZ and 2.0 mgl-1 2,4-D. The proliferated calli maintained their totipotency for more than one year (through subcultures). Two types of morphologically distinct calli (obtained via various callus induction treatments) were cultured on basal medium supplemented with 0.1 mgl-1 TDZ for shoot regeneration. Hundred percent regeneration frequency and maximum shoot bud regeneration (46 shoot buds/100 mg callus) were observed in calli induced on MS basal medium supplemented with 1.0 mgl-1 TDZ and 0.5 mgl-1 2,4-D (Callus line C10). PLB regeneration was observed in four callus lines producing shoot buds within one month, when transferred to fresh MS basal medium containing 0.1 mgl-1 TDZ. The regenerated shoots from different calli lines developed into complete plantlets when cultured on half-strength MS medium without any PGRs.

**Key words**: callus induction, orchid, plant growth regulators, protocorm-like-bodies, shoot regeneration

**INTRODUCTION**

*Bulbophyllum auricomum* Lindl., the royal flower of Myanmar is an epiphytic, deciduous, endangered orchid distributed in Thailand, Sumatra and Java. It flowers once a year and the blooms have an attractive perfume. It is mostly used commercially for cut-flower and ornamental purpose. Due to its ornamental value and commercial importance, the species has been over-exploited from the wild in the past and became endangered (Than et al. 2009, 2011). The need for mass propagation of selected elite genotypes of rare and economically important orchids has led to the development of several *in vitro* propagation methods (Arditti 2008). Plant tissues such as axillary buds, leaves, shoot tips, root tips, stem segments, protocorms, protocorm-like-bodies (PLB), rhizomes and pseudobulbs have been widely used as explants to induce callus and, subsequently to regenerate plantlets in different orchids species (Chen and Chang 2000a, Huan et al. 2004, Zhao et al. 2008, Tan et al. 2011).

Although callus exhibited great importance for mass propagation in many plant species, studies on callus induction in orchids are limited, perhaps due to their slow growth and necrotic tendency (Kerbauy 1984, 1991, Begum et al. 1994, Chang and Chang 1998, Roy and Banerjee 2001). Therefore, success of callus culture, where callus could be maintained for prolonged period through subculturing and subsequently regenerated into plantlet, has been limited to a few orchids (Ishii et al. 1998). Efforts to establish totipotent callus lines of orchids have been succeeded for some orchid species in recent years (Chen et al. 2000, Lu 2004, Zhao et al. 2008, Tan et al. 2011). However, there is no report on callus induction or indirect organogenesis in *B. auricomum*. The objective of the present study was to develop a reliable and efficient protocol for plant regeneration from callus cultures of *B. auricomum*.

**MATERIALS AND METHODS**

***Plant material***

Approximately three-month-old immature capsules of *B. auricomum* Lindl.were collected from wild populations found in the Yakhine Yoma mountain ranges in Myanmar during March, 2007. Immature capsule were surface sterilized with 15% teepol solution for 30 min followed by 1 min treatment with 70% ethanol and flamed for 10-15 sec. Sterilized capsules were dissected longitudinally and the seeds were extracted and aseptically cultured on modified KC (Knudson 1946) medium gelled with 0.75% w/v agar (Than et al. 2009). Within three to four weeks greenish PLB were collected and inoculated on MS (Murashige and Skoog 1962) nutrient medium and subcultured every two months before used as explants for callus induction.

***Initiation and establishment of callus culture***

Two to three mm PLB were bisected longitudinally and cultured in 20X150 mm culture tubes containing 20 ml of callus induction medium [half-strength macro- and microelements of MS supplemented with (in mgl-1) myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH2PO4 (170) and sucrose (20000) with the combination of 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) at 0, 0.1, 1.0 mgl-1 and 2,4-dichlorophenoxyacetic acid (2,4-D) at 0, 0.5, 1.0, 2.0 mgl-1]. Explants grown on basal MS nutrient medium without PGRs were used as a control. All these media were solidified with 0.75% w/v agar. The pH of the media was adjusted to 5.6 with KOH or HCl prior to autoclaving for 15 min at 121º C. Cultures were maintained in darkness at 24±1º C. The percentage of explants forming callus was scored after three and four months of culture (with monthly subculture). Growth of calli was expressed as GI (Growth Index, i.e. ratio between final fresh weight and the initial fresh weight of callus); inoculum in each treatment was ~50 mg of callus.

***Plant regeneration***

To evaluate potential of plant regeneration, ~ 100 mg (fresh weight) of callus from different lines were cultured on half-strength MS basal medium supplemented with 0.1 mgl-1 TDZ. Cultures were maintained under 16 h photoperiod (artificial fluorescent light 80 µmolm-2 s-1). The number of shoot buds per inoculum was recorded.

Regenerated shoots (~4.0 mm long) with 1-2 leaves were excised from the shoot clumps and transferred to the half-strength MS medium without PGRs for elongation and root induction. Data were recorded after two months of culture.

***Statistical analyses***

Twenty explants were taken for each treatment and each experiment was repeated thrice. Tabulated results were analyzed using one-way ANOVA (Sokal and Rohlf 1987) and statistical differences between mean values were analyzed using the standard error (S.E) and Duncan’s Multiple Range Test with the Statistica Software v 5.0 (StatSoft 1995).

**RESULTS**

***Callus induction from PLB segments***

Optimum (100%) survival was observed when explants (bisected PLB segments) were cultured in medium containing either in 0.1 mgl-1 TDZ combined with 2,4-D (0-1.0 mgl-1) or in 1.0 mgl-1 TDZ combined with 2,4-D (0-0.5 mgl-1) supplemented MS medium (Table 1). These results demonstrate that the absence or low concentrations of 2,4-D in combination with TDZ (0.1-1.0 mgl-1) were most effective in avoiding explant necrosis. Callus was induced on PLB segments within one month of inoculation. Two types of morphogenetically distinct calli were obtained from PLB explants. One type of callus, yellowish and friable, was induced on medium supplemented with 2,4-D alone (Fig. 1a, Table 1). The other type, yellowish green and friable, was induced on MS medium supplemented with different concentrations and combinations of TDZ and 2,4-D (Fig. 1b, Table 1). It was observed that high concentration of 2,4-D enhanced frequency of callus induction (77%). Callus induction frequency was highest in MS medium supplemented with 1.0 mgl-1 TDZ and 2.0 mgl-1 2,4-D after 3-4 months of culture.

***Callus proliferation and PLB induction in callus cultures***

Calli derived from PLB explants were subcultured on half-strength MS basal medium supplemented with different combinations of TDZ and 2,4-D. Basal medium supplemented with 0.1 mgl-1 TDZ and 2.0 mgl-1 2,4-D was found to be most suitable for proliferation of callus (Growth Index=9.46±0.91) as compared to other combinations tried (Table 2). The proliferated calli maintained their totipotency for more than one year (through subcultures). However, the calli cultured on medium containing 1.0 mgl-1 TDZ were proliferated as evident from the increase in weight but gradually became necrotic after 2 months of culture. The calli cultured without PGRs or basal medium supplemented with 0.1 mgl-1 TDZ and 0.5 mgl-1 2,4-D were eventually turned brown and necrotic. Callus proliferated but occasionally produced PLB when cultured on basal medium containing either 0.1 mgl-1 TDZ alone or 1.0 mgl-1 TDZ in combination with 0.5 mgl-1  2,4-D.

***Shoot regeneration and multiplication from callus cultures***

The regeneration frequency and shoot bud induction were affected by the concentrations and combinations of callus induction medium. Initially, two types of morphologically distinct calli (raised in different callus induction media) grew well and gradually turned green. Calli from C2, C3, C4 and C11 callus lines gradually turned brown and finally became necrotic after 3 weeks of culture (Table 3). The C8 callus line was green and compact, but neither shoot differentiation nor PLB regeneration was observed in this line. Globular structures with shoot meristem developed in calli lines C5, C7, C10 and C12 (Fig. 1c). These globular structures developed into PLB after 2 weeks of culture (Fig. 1d). The regenerated PLB produced shoot buds within one month when transferred to the basal medium containing TDZ (0.1 mgl-1) (Fig. 1e). The 100 percent regeneration frequency was achieved in calli induced on MS basal medium supplemented with 1.0 mgl-1 TDZ and 0.5 mgl-1 2,4-D (Callus line C10). The rate of shoot bud induction was affected by duration of callus culture on the shoot regeneration medium. The optimum shoot bud formation (46 shoot buds/100 mg callus) was observed in monthly subcultured callus line C10, after 4 months of culture.

The regenerated shoots from different callus lines grew well when cultured on half-strength MS medium without PGRs. Root induction was noted in regenerated shoots within one month of culture (Fig. 1f). There was no significant difference in the shoot length and number of root formation among different callus lines (Table 4). The optimum root length (8.1±0.6 mm) was noted in C10 callus line. In general, the regenerated shoots when isolated from different callus lines were developed into complete plantlets within 2 months of culture and additional shoots were induced from *de novo* generated PLB (Fig. 1g).

**DISCUSSION**

Callus induction from PLB segments of *B. auricomum* was studied using various concentrations and combinations of TDZ and 2,4-D as reported in other orchid species by Chang and Chang (1998), Lin et al. (2000), Chen et al. (2000), Lee and Lee (2003) and Lu (2004). In this study, callus induced within one month of PLB explant culture as reported in *Cymbidium* (Huan and Tanaka 2004). On the contrary, longer time (12–18 months) was required for callus induction from pseudobulb sections, rhizomes, and roots of seed-derived plantlets in *Cymbidium ensifolium* (Chang and Chang 1998). In *Phalaenopsis*, callus induction from shoot-tips excised from floral buds was obtained after 7 months (Tokuhara and Mii 2001). The results clearly indicated the crucial role of exogenous PGRs on callus induction. Callus induction in orchids requires PGR supplementations in the basal medium (Chen and Chang 2000a, Lin et al. 2000, Roy and Banerjee 2003, Huan et al. 2004). Callus induced in protocorm segment of *Cypripedium formosanum* (Lee and Lee 2003) and shoot apex of *Paphiopedilum* (Stewart and Button 1975) cultured on only 2,4-D supplemented medium failed to survive during subcultures. An auxin is generally required for the induction of callus from explants, however, presence of a cytokinin may not be essential to differentiate callus especially from explants of monocotyledons (George et al. 2008).

Protocorm segment in *Paphiopedilum* (Lin et al. 2000), *C. formosanum* (Lee and Lee 2003), and PLB segments in *Cymbidium* (Huan et al. 2004) did not show induction of callus in the presence of TDZ alone, as observed in the present study on *B. auricomum*. Stimulatory effects of TDZ in combination with 2,4-D for callus induction have also been reported in *Phalaenopsis* (Chen et al. 2000), *Paphiopedilum* (Lin et al. 2000), *C. formosanum* (Lee and Lee 2003), *Cymbidium* Twilight Moon ‘Day light’ (Huan et al. 2004) and *Pleione formosana* (Lu 2004). Thus it can be concluded that many species of orchids require supplementary cytokinin along with auxin for optimum response of callus induction and in some cases to prevent necrosis of callus (Ignacimuthu et al. 1999).

In orchids, successful reports on plant regeneration from totipotent callus have been limited due to slow growth rate and a tendency to become necrotic (Kerbauy 1991). Chang and Chang (1998) reported callus induction from several kinds of explants in a terrestrial orchid, *Cymbidium ensifolium*. Callus induction from these explants requires high levels of PGRs and callus growth was slow, difficult and time consuming. In recent years, the callus induction frequency in orchids have been reported using different source of explants, for example, 25% from root tips (Chen and Chang 2000a), 66 % (Roy and Banerjee 2003) and 46 % (Roy et al. 2007) from shoot tips, 53% from PLB segments (Huan et al. 2004), 50% from seed-derived protocorms (Lu 2004), and 82% from protocorm segments (Zhao et al. 2008). In the present study, hundred percent callus induction have been obtained from PLB explants cultured on basal medium supplemented with 1.0 mgl-1 TDZ and 2.0 mgl-1 2,4-D .

In orchids, there are only few reports on callus induction and differentiation in callus cultures. In recent years, successful establishment of calli and regeneration of plantlets through PLB formation have been reported in some orchid species (Chen and Chang 2000a, Lu 2004). The micropropagation through indirect PLB formation may be effective for the high frequency of somatic embryo formation and efficiency of embryo conversion into plants (Zhao et al. 2008). This type of morphogenesis has been reported in a number of orchid species (Colli and Kerbauy 1993, Chen and Chang 2000a, Lin et al. 2000, Lee and Lee 2003, Roy and Banerjee 2003, Huan and Tanaka 2004), and suggested that the process of somatic embryogenesis is a part of the early steps of PLB regeneration in orchids (Kerbauy 1984, Begum et al. 1994, Ishii et al. 1998, Chen and Chang 2000a, Huan et al. 2004, Zhao et al. 2008). Thus, the regeneration of PLB along with subsequent differentiation of shoot shows great potential as an attractive method for the mass production of this economically important orchid.

The efficacy of low level of TDZ over other cytokinins for direct PLB formation was earlier reported in *Doritaenopsis* (Park et al. 2003). TDZ has been reported to be effective in inducing morphogenesis in several orchids, such as increased protocorm proliferation rate, enhanced PLB formation (Ernst 1994), enhanced shoot regeneration and proliferation (Chen and Piluek 1995, Chen and Chang 2000b) and direct somatic embryogenesis (Chen et al. 1999, Chen and Chang 2000b, 2001, 2002). However, TDZ inhibited shoot proliferation and root induction in *Paphiopedilum* (Huang et al. 2001). In the present study, TDZ was found to be effective in shoot regeneration from callus cultures.

In *B. auricomum*, approximately 46 shoot buds could be induced from 100 mg callus on basal medium containing 0.1 mgl-1 TDZ. Lin et al. (2000) have demonstrated that approximately 100 mg of protocorm-derived callus induced 3-7 shoot buds in optimal conditions in *Paphiopedilum*. Induction of 10.2 shoot buds was obtained from vegetative buds of floral stalk of *Phalaenopsis* in presence of 1.0 mgl-1 TDZ (Chen and Piluek 1995). Hence, shoot bud regeneration frequency in *B. auricomum* was much higher than reported from any other orchid species.

Depending on the explant type and cultural conditions, direct somatic embryogenesis is the usual pathways in a number of orchid species (Colli and Kerbauy 1993), while in others an intermediary callus appeared to be a prerequisite for PLB regeneration (Chang and Chang 1998, Ishii et al. 1998). Therefore, to make use of the morphogenic potential of callus as an effective system of micropropagation, it is essential to develop a reliable technique for callus induction. The protocol demonstrated in the present study for callus induction, proliferation and shoot regeneration in *B. auricomum* is simple, efficient and could be applied for mass propagation of this valuable species.

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**Legend of Figure**

Fig. 1. Callus derived from *in vitro* culture of PLB segments of *Bulbophyllum auricomum*

(a) yellowish friable callus grown on half-strength MS medium supplemented with 2.0 mgl-1 2,4-D, Bar 1.3 mm; (b) yellowish green friable callus grown on half-strength MS medium supplemented with 1.0 mgl-1 TDZ and 2.0 mgl-1 2,4-D, Bar 1.2 mm; (c) shoot-bud like structures (arrows) on the surface of callus grown on half-strength MS medium supplemented with 0.1 mgl-1 TDZ, Bar 0.2 mm; (d) PLB like structures (arrows) appeared on the surface of the culture grown on half-strength MS medium supplemented with 0.1 mgl-1 TDZ after 2 weeks, Bar 0.3 mm; (e) Differentiation of shoots in cluster, Bar 1.4 mm; (f) rooted plantlet on half-strength MS basal medium, Bar 3.4 mm; (g) new shoots (arrows) appeared from *de novo* generated PLBs, Bar 2.4 mm.

Table 1. Effects of different concentrations and combinations of TDZ and 2,4-D on callus induction from PLB segments of *B. auricomum* grown under dark condition.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| TDZ (mgl-1) | 2,4-D (mgl-1) | Percentage of explants showing callus induction \* | | Nature of callus |
| 90 days | 120 days |
| 0 | 0 | 0a | 0a | Direct shoot bud induction, no callus formation |
| 0 | 0.5 | 29±0.37ab | 29±0.37ab | Yellowish friable |
| 0 | 1.0 | 50±0.24b | 56±0.20bc | Yellowish friable |
| 0 | 2.0 | 67±0.20b | 77±0.31c | Yellowish friable |
| 0.1 | 0 | 0a | 0a | Direct shoot bud induction, no callus formation |
| 0.1 | 0.5 | 22±0.20ab | 22±0.20ab | Yellowish green friable |
| 0.1 | 1.0 | 20±0.36ab | 20±0.36ab | Yellowish green friable |
| 0.1 | 2.0 | 50±0.38b | 50±0.38bc | Yellowish green friable |
| 1.0 | 0 | 0a | 0a | Direct shoot bud induction, no callus formation |
| 1.0 | 0.5 | 10±0.22a | 20±0.20ab | Yellowish green friable |
| 1.0 | 1.0 | 13±0.25a | 33±0.31abc | Yellowish green friable |
| 1.0 | 2.0 | 94±0.37c | 100±0.25d | Yellowish green friable |

\*Mean of 20 explants ± standard error (S.E). Values followed by different superscript letters within a column are significantly different according to Duncan’s multiple range tests at 1% probability level.

Table 2. Effects of different concentrations and combinations of TDZ and 2,4-D on PLB-derived callusproliferation in *B. auricomum* after 90 days grown *in vitro* under dark condition.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Callus Lines | TDZ (mgl-1) | 2,4-D (mgl-1) | Growth index (GI) **\***  (n=20) | Phenotype of callus |
| C1 | 0 | 0 | 0a | Necrotic |
| C2 | 0 | 0.5 | 6.87±0.30cd | Yellowish friable |
| C3 | 0 | 1.0 | 6.92±0.16d | Yellowish friable |
| C4 | 0 | 2.0 | 7.67±0.26d | Yellowish friable |
| C5 | 0.1 | 0 | 4.37±0.39b | PLBs regenerated from callus |
| C6 | 0.1 | 0.5 | 0a | Necrotic |
| C7 | 0.1 | 1.0 | 7.52±0.63d | PLBs regenerated from yellowish green friable callus |
| C8 | 0.1 | 2.0 | 9.46±0.91e | Yellowish green friable |
| C9 | 1.0 | 0 | 7.04±0.26d | Necrotic |
| C10 | 1.0 | 0.5 | 7.50±0.22d | PLBs regenerated from callus |
| C11 | 1.0 | 1.0 | 5.70±0.55bc | Yellowish green friable |
| C12 | 1.0 | 2.0 | 7.11±0.26d | PLBs regenerated from yellowish green friable callus |

**\*** Mean ± standard error (S.E). Values followed by different superscript letters are significantly different according to Duncan’s multiple range tests at 1% probability level.

Table 3. Regeneration potential of different calli lines induced from PLB segments of *B. auricomum* in the presence of different concentrations and combinations of PGRs.\*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Callus Lines | MS basal medium containing (mgl-1) | | Regeneration frequency % | No. of shoot buds/100 mg callus \*\* | |
| TDZ | 2,4-D | 60 days | 120 days |
| C2 | 0 | 0.5 | 0 | 0 a | 0 a |
| C3 | 0 | 1.0 | 0 | 0a | 0 a |
| C4 | 0 | 2.0 | 0 | 0 a | 0 a |
| C5 | 0.1 | 0 | 40 | 2.5±0.29b | 11.0±0.55b |
| C7 | 0.1 | 1.0 | 30 | 0.5±0.12 a | 1.5±0.12 a |
| C8 | 0.1 | 2.0 | 0 | 0 a | 0 a |
| C10 | 1.0 | 0.5 | 100 | 4.5±0.52c | 45.5±2.48c |
| C11 | 1.0 | 1.0 | 0 | 0 a | 0 a |
| C12 | 1.0 | 2.0 | 10 | 0 a | 1.5±0.12 a |

\*Cultures were maintained at 16 hr photoperiod.

\*\*Mean number of shoot buds per 100 mg fresh weight of callus. The mean of 20 replicates ± standard error (S.E). Values followed by different letters within a column are significantly different according to Duncan’s multiple range tests at 1% probability level.

Table 4. Shoot development and root induction in regenerated shoots from different calli lines (n=20)cultured on half strength MS medium.\*

|  |  |  |  |
| --- | --- | --- | --- |
| Callus lines | Mean no. of root/plant \*\* | Root length (mm) \*\* | Shoot length (mm) \*\* |
| C5 | 2.9±0.4 | 4.0±0.7 | 12.0±1.0 |
| C7 | 3.0±0.1 | 6.3±0.4 | 14.2±0.6 |
| C10 | 3.8±0.4 | 8.1±0.6 | 17.4±2.7 |
| C12 | 2.4±0.3 | 4.0±0.3 | 11.4±1.0 |

\*Cultures were maintained at 16 hr photoperiod.

\*\* The mean ± standard error (S.E).

**Response to reviewer’s questions**

**Reviewer 1**

Q: Concentration of teepol solution and duration of the treatment?

A: 15% teepol solution for 30 minutes

**Reviewer 2**

Q: What does cent percent mean?

A: Cent percent means hundred percent.

Q: How did you determine age? Did you tag flowers at the time that pollen was dehisced? did

you conduct hand pollinations? Did you make an educated guess?

A: *B. auricomum* flowers in December and immature capsule were collected in March. No hand hand pollination were conducted.

Q: Did this medium contain any sugar or vitamins?

A: Modified KC (Knudson 1946) medium contains 20 gm/l sucrose without vitamins.

Q: Was the control medium full-strength or ½-strength MS?

A: The control/basal medium containing half-strength macro- and microelements of MS

supplemented with (in mgl-1) myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5),

thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH2PO4 (170) and sucrose (20000)

**Reviewer 3**

Q: Please indicate it is wild material or if breeding has already been undertaken, it is probably

an outbreeding species?

A: It is wild material.

Q: It is quite uncommon to propagate plants, and especially orchids via callus. The authors must describe their intentions why to undergo this way instead of using axillary shoot formation or PLB proliferation. If callus cultures are going to be used for mass propagation as said by the authors, experiments to test the stability of the regenerated plants are urgently needed and have to be discussed.

A: Different source of explants such as seed, seed-derived shoot (Than et al. 2009), pseudobulb (Than et al. 2011), leaf and stem segment (unpublished) have been used for mass

propagation. We agree , for propagation the stability of regenerated plants must be determined and will be done.

Q: It is absolutely unclear for the readers when the regenerating structures that arise from callus cultures were named PLBs, when shoots and when even somatic embryos! Please avoid this confusion and explain and use all terms properly.

A: Corrections done .

Q: Materials: When were the protocorms used for the experiments? (how long after sowing?)

A: Six-week-old protocorms developed into shoots when transferred to Modified Knudson C medium (Than et al. 2009). Within three to four weeks, shoot-derived greenish PLB were collected and inoculated on MS (Murashige and Skoog 1962) nutrient medium and subcultured every two months before used as explants for callus induction.

Q: There are several terms that are either not used in the correct way: The authors did not

induce callus from PLBs as stated in many many places in the text, but from protocorms.

This is not acceptable at all.

A: Corrected in revised MS.

Q: Did you really use only 2 g/l sucrose???

A: Corrected , used 20g/l sucrose.

Q: Table 1: How can more explants form callus than explants do survive?

A: Table 1: Explants which induce callus are expressed as percentage of callusing.

Q: Are the values in the tables the means of the three replications of each experiment?

A: Yes.

**Response to Editors letter**

**Dear Sir,**

**We have tried to respond to all your queries and also attend to reviewers comments. Hope the revised MS will be accepted for publication. The inadvertent delay was due to the fact that Ms Myo Ma Ma Than is in Myanmar and it was away for Holy Haz pilgrimage.**

**Sincerely**

**Authors**