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# Asymbiotic seed germination and mass multiplication of *Taprobanea spathulata* (L.) Christenson (Asparagales: Orchidaceae): a medicinally important epiphytic orchid

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**Abstract**. An effective protocol was developed for asymbiotic seed germination and mass multiplication of *Taprobanea spathulata* (L.) Christenson a medicinally important epiphytic orchid. Different types of media such as MS, $B_5$, Mitra, KCM and LO media was tested for seed germination and protocorm development. Half strength MS medium was found to be best for seed germination (92.73 $\pm$ 1.18%) and protocorm development. Protocorms were cultured on half strength MS medium with different growth hormones such as BAP, KIN, NAA, IAA (0.5-2.0 mg/L) either individually or in combination. Organic additives such as peptone, coconut water and tomato juice were tested either individually or in combination with KIN for multiple shoot induction. KIN (1.0 mg/L) along with IAA (1.0 mg/L) was found to be best in producing multiple protocorms (22.40 $\pm$ 1.33) with 1.60 $\pm$ 0.24 number of roots. The organic additives peptone at 0.5% individually (14.64 $\pm$ 0.312) and 0.125% in combination with KIN (0.5 mg/L) was effective in producing 16.84 $\pm$ 0.639 multiple protocorms. Both NAA and IAA at 0.5 mg/L individually were effective for inducing healthy roots. Plantlets with well-developed leaves and roots were transplanted to vermiculite for acclimatization. The survival rate was 67%.

**Keywords**: Seed germination, Multiple protocorms, IAA, Peptone, Activated charcoal, Vermiculite.

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## Introduction

Orchidaceae is amongst the most diverse family of the flowering plants consisting of 35,000 species under 880 genera (Singh et al., 2007) occurring widely in the humid tropical forests of India, Sri Lanka, South Asia, South and Central America and Mexico. Nearly 1,331 species of orchids in 140 genera dwell in India, with Himalayas as their main habitat and others scattered in Eastern and Western Ghats. In Peninsular India about 371 species have been reported (Saranya et al., 2012). Romans and Greeks named the plant as “orchis” a (Greek) meaning testis which refers to the orchid’s prominent tubers. Orchids play a significant role in traditional systems of medicine, because they possess large quantity of alkaloids, flavonoids, glycosides and other phytochemicals (Gutierrez, 2010).

Flower juice of *Vanda coerulea* Griff. *ex* Lindl. is used as eye drops against glaucoma, cataract and blindness (Anonymous, 1992), the leaves are used externally in skin diseases and leaf juice is used in diarrhoea and dysentery (Hossain, 2011). In *Vanda testacea* (Lindl.) Rchb.f. (1877) the crushed leaf is applied on cuts and wounds and boiled leaves are used as ear ache (Nadkarni, 1999). The therapeutic values of 58 orchids have been studied by Harshitha Kumari et al. (2012). *Vanda* is

the most prominent orchid among several epiphytic orchids. In the last three decades, Vandas are popular in floricultural industries and are cultivated outdoors or in green house in the warm regions of the world. This prominent group of orchid genera includes 70 species (Bose et al., 1999) which are monopodial epiphytes; eventhough some are lithophytes.

*Vanda spathulata* (L.) Spreng., was the first *Vanda* species ever described back in 1703 in the 'Hortus Indicus Malabaricus' as Ponnampou-maravara. Later, Linnaeus named it *Epidendrum spathulatum* in 1753 and it did not get its present name until 1826. *T. spathulata* is commonly known as Svarna-pushpa bandaa or baandaa or Mara Vazha (means tree-top plantain).

*Taprobanea spathulata* (L.) Christenson is a perennial herbaceous, epiphytic orchid species endemic to peninsular India including Tamil Nadu, Kerala, Karnataka and Maharashtra and Sri Lanka. This species have large golden yellow flowers with size ranges from 4-5 cm, bloom during October-December.

The leaf and flower powder of *T. spathulata* cures nervous system disorders and nervous debility (Shanavaskhan et al., 2012). Dried flowers were powdered and given for asthma (Guha Bakshi Sensarma and Pal, 2001), depression, maniac troubles scorpion sting (Shanavaskhan et al., 2012), skin diseases and diarrhoea (Madhi and Chakrabarti, 2009). The juice of this plant is given to temper the bile, abate frenzy and as a liver tonic (Pullaiah, 2006; Dasari et al., 2013). *T. spathulata* is a rare orchid in Madukkarai Hills, Coimbatore (Jayanthi et al., 2011), but Basha et al. (2012) reported it as an endangered species and its distribution is restricted to narrow pocket due to anthropogenic activity (Miria et al., 2012).

Careless collection of these species has led to serious genetic and ecological erosion; many orchids have been listed as endangered species (Machaka-Houri et al., 2012). Therefore, tissue culture techniques have been preferred. This is due to its tremendous promise in improving their quality and quantity through mass propagation of several commercially important orchids and new hybrid or a variety within a short time (Goh et al., 1990; Chen and Chang, 2000, 2004) and it is proved to be useful for the plants which are difficult to propagate using conventional techniques (Fay, 1994). In the present study, rapid and efficient method for asymbiotic seed germination and mass propagation from the seeds was developed for *T. spathulata.*

## Materials and methods

### Source of plant material

Undehisced green capsules of *T. spathulata* were collected from the Madukkarai Hill, which is located at 10.9° N and 76.97° E along the hill sides of the Southern Western Ghats. An authentic sample was identified by BSI (Botanical Survey of India), Southern Circle, Coimbatore, India, and deposited in the herbarium of BSI.

### Explant and mode of sterilization

The green pods (7 cm) were harvested from actively growing healthy plants and washed thoroughly under running tap water followed by 1% (v/v) detergent solution of 'Teepol' for 5 min. The explants were then rinsed repeatedly with sterile distilled water and then treated with a fungicide Bavistin (1% w/v) for 10 min and washed thoroughly with sterile distilled water. The explants were then surface sterilized with 0.01% (w/v) $HgCl_2$ solution for about 5 min followed by five to six repeated washes with sterilized double-distilled water in order to remove traces of sterilant. Then the pods were dipped in 80% ethanol for 1 min and flamed and aseptically inoculated in to pre-cooled autoclaved media.

### Culture media and growth conditions

Lindemann orchid medium (1970), Knudson C Orchid Morel medium (1946), Gamborg's medium (1968) and Mitra orchid medium (1986) were procured from "hi-media" and MS (1962) (full, half and quarter strength) was prepared fresh which involves individual preparation of the stock

solutions (macronutrients, micronutrients, iron, potassium iodide and vitamins). The macro and micro nutrients were weighed accurately using electronic weighing balance (Electronic balance, ER-182A).

**Effect of auxin and cytokinins on multiple shoot induction**

After sterilization, the seeds were inoculated on half strength MS medium supplemented with various growth regulators such as BA, KIN, NAA and IAA (0.5, 1.0 and 2.0 mg/L) either individually or in combinations. The above medium was supplemented with 3% (w/v) sucrose, activated charcoal (0.5-1.0 g/L) and solidified with 0.8% agar. The pH was adjusted to 5.8.

**Effect of combinations of growth regulators on multiple shoot induction**

For multiple shoot induction, seed derived protocorms (0.5-1.0 mm) were cultured on half strength MS medium supplemented with different concentrations (0.5, 1.0 and 2.0 mg/L) of BA, KIN, NAA and IAA either individually or in combinations. In addition organic additives such as peptone (0.125, 0.25 and 0.5%), coconut water and tomato juice (5, 10 and 15%) were used alone or in combination with 0.5 mg/L of KIN for multiple shoot induction.

**Effect of organic additives**

Various organic additives such as peptone (0.125, 0.25 and 0.5%), coconut water (5, 10 and 15%) and tomato juice (5, 10 and 15%) were added into the medium before adjusting the pH.

**Seed viability test**

Seed viability was tested according to Vellupillai et al. (1997). For counting the viability percentage, seeds obtained from the fresh capsules were stained with 1% (w/v) 2,3,5-triphenyl tetrazolium chloride (pH 7.0) in darkness overnight and then were observed under light microscope. Red colour in the embryo indicated seed viability.

**Rooting**

The well-developed plantlets were transferred to half strength MS medium supplemented with NAA or IAA at different concentrations (0.5, 1.0 and 2.0 mg/L) to induce rooting.

**Hardening**

Plantlets with well developed roots were removed and gently washed under sterile distilled water to remove the adhering medium. Subsequently, they were transferred to plastic paper cups containing sterile vermiculite. Initially the plantlets were maintained at $25 \pm 2$ °C under a 16 h photoperiod of 50-60 $\mu mol/m^{-2}/s^{-2}$ flux density for few weeks to observe its growth.

**Statistical method**

The data from five replicates in each experiment were subjected to analysis of variance (ANOVA) and the means were performed by the Duncan's multiple range test (DMRT), using the SPSS 20 (SPSS Inc., Chicago, IL, USA).

## Results

*T. spathulata* has scandent stem of about 120 cm long which have leaves of flat, ovate or linear oblong, obtuse or subacute apex, oblique and entire of emarginate, 10 cm long and 3 cm width. The inflorescence of *T. spathulata* is 45 cm long with few to many flowered raceme having erect leaves and scapes often marked with blood red spots. Its golden yellow flowers are more than 3 cm across, sepals and petals spathulately oblong, flat, lip clawed as long as sepals, very small, midlobe subordicular (Figure 1).

**Asymbiotic seed germination**

The seeds (Figure 2, a) were sowned on various basal media like Mitra orchid (M), Murashige and Skoog (MS), Half S (1/2 strength MS), Quarter MS (1/4 strength MS), Lindemann orchid (LO), Knudson C Orchid Morel (KCM) and Gamborg's ($B_5$) (Figure 3, a-g). The process of seed germination and development of embryos were divided into

four stages such as spherule stage, protocorm stage, shoot stage and rhizoid stage. During germination the embryos imbibed water through testa and were found to be swollen (Figure 2, b). The undifferentiated embryo emerged from the testa called spherule (Figure 2, c) which developed into green protocorm with shoot meristem at the apex and rhizoids on the opposite side (Figure 2, d).


**Figure 1.** *T. spathulata*. (a) Habit (with pod). (b) Inflorescence.

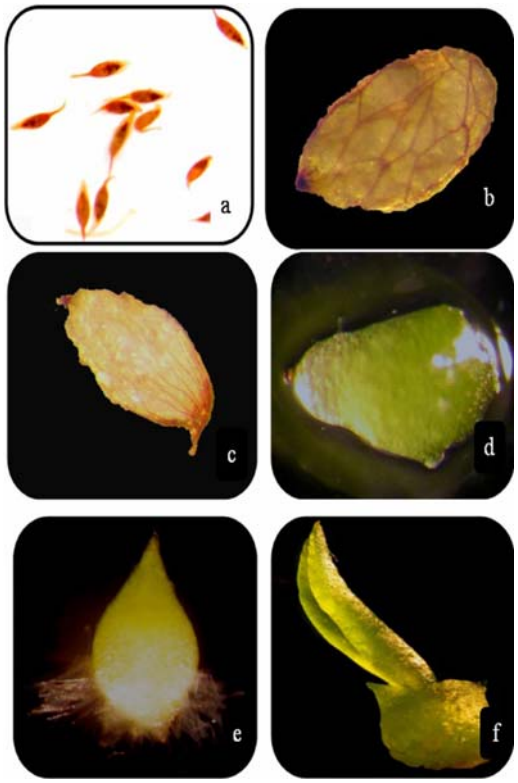


**Figure 2**. Seed germination in *T. spathulata* (viewed under stereo microscope). Enlarged view of (a) Seed at the time of inoculation; (b) Swollen embryo; (c) Embryo emerging from the seed coat; (d) Formation of green protocorm; (e) Protocorm with shoot apex and rhizoids; (f) Formation of first leaf.

The highest percentage of germination was observed in 1/2 strength MS (Figure 3, f) followed by 1/4 strength MS (Figure 3, e), $B_5$, M, MS, KCM and LO (Figure 4) and the organogenesis with respect to time was recorded (Figure 5). But in LO medium at spherule stage, the further growth was arrested and embryos were dehydrated. In M, KCM and $B_5$ media hyper-hydrated glassy protocorms were formed which did not favour further development. In different strengths of MS (1/4 strength, 1/2 strength and full strength), the protocorms developed well and further produced seedlings. However, the percentage of seed germination ($92.73 \pm 1.18\%$) (Table 1) was found to be best in 1/2 strength MS. Hence, 1/2 strength MS was selected to carry out further studies on protocorm multiplication and seedling growth.

**Effect of individual growth regulators.** Seed derived protocorms (16 weeks old) when cultured on 1/2 strength MS medium devoid of growth regulators (GR) formed a single shoot. The above medium when supplemented with cytokinins such as BA and KIN and auxins such as IAA and NAA individually at different concentrations produced multiple protocorms. The effect of growth regulators on growth and differentiation of protocorm was studied (Table 2**)**. Among different cytokinins tested, KIN (0.5 mg/L) was found to be superior in inducing maximum number of multiple protocorms ($12.00 \pm 1.37$) with less shoot length (Figure 6, a and b) and BAP at 2.0 mg/L enhanced the shoot length ($1.74 \pm 0.21$ cm). In the case of auxins, both IAA and NAA were quite effective in seedling

**Table 1**. Effect of various media on asymbiotic seed germination in *T. spathulata* seed.

| Medium | % of germination (at 8th week) Mean ± S.E. | No. of protocorms Mean ± S.E. |
|---|---|---|
| 1/4 MS | 68.34 ± 3.926$^{c}$ | 112.20 ± 3.44$^{c}$ |
| 1/2 MS | **92.73 ± 1.180$^{a}$** | **178.20 ± 3.82$^{a}$** |
| MS | **90.78 ± 1.584$^{a}$** | 151.80 ± 3.16$^{b}$ |
| M | 76.05 ± 2.416$^{bc}$ | 43.40 ± 2.13$^{d}$ |
| KCM | 51.47 ± 4.204$^{d}$ | 18.40 ± 2.74$^{e}$ |
| LO | 48.83 ± 3.325$^{d}$ | 0.00 ± 0.00$^{f}$ |
| B5 | 85.96 ± 6.063$^{ab}$ | 118.80 ± 1.77$^{c}$ |

*****Mean ± Standard error followed by the common letter are not significant at the P ≤ 0.05% level by DMRT.

**Table 2**. Effect of growth regulators on formation of multiple protocorms.

| Growth regulator (mg/L) | | | | Shoot length Mean ± S.E (cm) | No. of multiple protocorms/ explant Mean ± S.E | Root number Mean ± S.E | Root length Mean ± S.E (cm) |
|---|---|---|---|---|---|---|---|
| BA | Kn | IAA | NAA | | | | |
| 0 | 0 | 0 | 0 | 1.22 ± 0.05$^{b}$ | 1.00 ± 0.00$^{d}$ | 1.40 ± 0.07$^{ab}$ | **1.02 ± 0.04$^{a}$** |
| 0.5 | - | - | - | 0.99 ± 0.09$^{bcd}$ | 1.00 ± 0.00$^{d}$ | 0.80 ± 0.20$^{c}$ | 0.16 ± 0.05$^{de}$ |
| 1 | - | - | - | 0.93 ± 0.14$^{bc}$ | 1.00 ± 0.00$^{d}$ | 0.20 ± 0.20$^{d}$ | 0.10 ± 0.10$^{de}$ |
| 2 | - | - | - | **1.74 ± 0.21$^{a}$** | 5.20 ± 0.86$^{b}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ |
| - | 0.5 | - | - | 0.66 ± 0.04$^{d}$ | **12.00 ± 1.37$^{a}$** | 1.50 ± 0.17$^{ab}$ | 0.41 ± 0.08$^{bc}$ |
| - | 1 | - | - | 0.78 ± 0.05$^{cd}$ | 4.80 ± 0.58$^{bc}$ | 0.80 ± 0.20$^{d}$ | 0.13 ± 0.04$^{de}$ |
| - | 2 | - | - | 0.78 ± 0.08$^{cd}$ | 3.40 ± 1.16$^{bcd}$ | 0.20 ± 0.20$^{d}$ | 0.13 ± 0.13$^{cd}$ |
| - | - | 0.5 | - | 1.02 ± 0.06$^{bc}$ | 1.26 ± 0.26$^{d}$ | **1.85 ± 0.10$^{a}$** | 0.49 ± 0.08$^{b}$ |
| - | - | 1 | - | 0.99 ± 0.02$^{bcd}$ | 1.00 ± 0.00$^{d}$ | 1.07 ± 0.14$^{bc}$ | 0.51 ± 0.02$^{b}$ |
| - | - | 2 | - | 0.96 ± 0.08$^{bcd}$ | 1.00 ± 0.00$^{d}$ | 1.30 ± 0.20$^{abc}$ | 0.42 ± 0.04$^{bc}$ |
| - | - | - | 0.5 | 0.93 ± 0.04$^{bcd}$ | 4.20 ± 1.62$^{bc}$ | 1.50 ± 0.03$^{ab}$ | 0.49 ± 0.05$^{b}$ |
| - | - | - | 1 | 1.11 ± 0.15$^{bc}$ | 2.60 ± 1.02$^{cd}$ | 1.65 ± 0.10$^{bc}$ | 0.43 ± 0.06$^{bc}$ |
| - | - | - | 2 | 1.14 ± 0.06$^{b}$ | 1.00 ± .00$^{d}$ | **1.80 ± 0.37$^{a}$** | 0.26 ± 0.02$^{cd}$ |

*****Mean ± Standard error followed by the common letter are not significant at the P ≤ 0.05% level by DMRT

development. NAA (2.0 mg/L) and IAA (0.5 mg/L) (Figure 6, e) enhanced the root number which ranges from 1.80 ± 0.37 and 1.85 ± 0.10 respectively, but growth regulator free medium showed highest root length (1.02 ± 0.040 cm) (Figure 6, f).

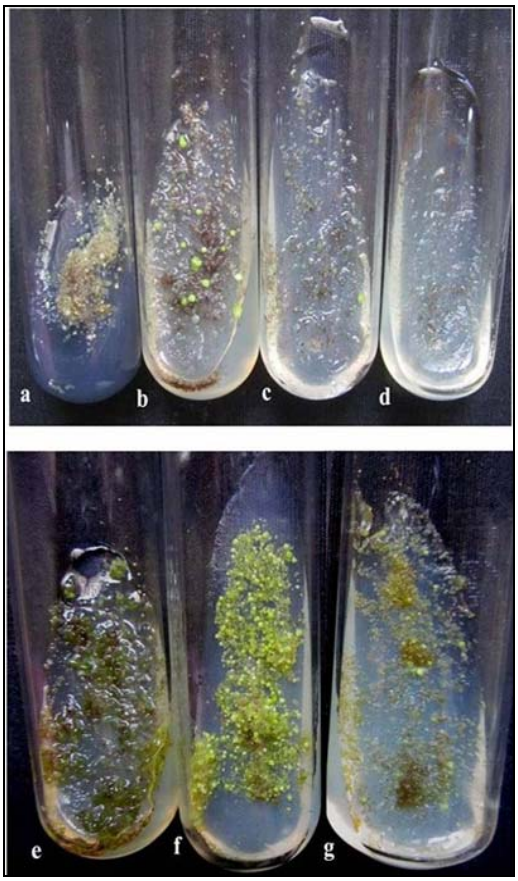


**Figure 3**. Seed germination on (a) Mitra orchid medium; (b) MS (full strength) medium; (c) Lindemann orchid medium; (d) Knudson C orchid Morel medium; (e) Gamborg's medium; (f) MS (half strength) medium; (g) MS (quarter strength) medium.

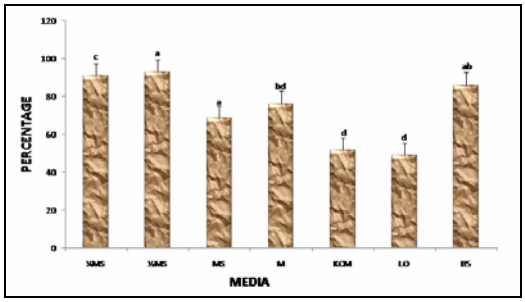


**Figure 4**. Germination of *T. spathulata* seeds on different media (at 8th week).

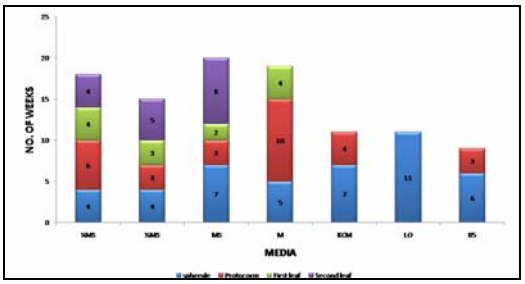


**Figure 5**. Germination of seed representing the duration of different developmental stages.

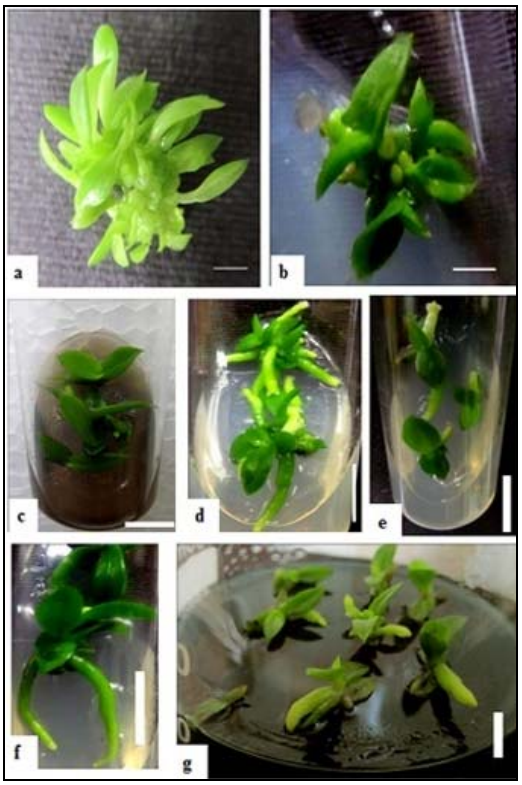


**Figure 6**. (a and b) Multiple protocorms developed on 1/2 MS + Kn (0.5 mg/L) (bar = 1 cm); (c) Development of shoot on 1/2 MS + BA (2 mg/L) with IAA (2 mg/L) combination (release of phenols into the medium) (Bar = 2.5 cm); (d) Multiple shoot and root development in Kn (1 mg/L) + IAA (1 mg/L) (Bar = 2 cm); (e) Roots developed on 1/2 MS + NAA (0.5 mg/L) (Bar = 1.5 cm); (f) Development of long roots in growth regulator free ½ MS medium (Bar = 3 cm); (g) Plantlets grown on activated charcoal (0.5 mg/L) containing medium (Bar = 2 cm).

**Effect of combination of growth regulators**. The effect of cytokinins (BA and KIN) in combination with auxins (IAA and NAA) at different concentrations (0.5, 1.0 and 2.0 mg/L) were tested to examine

their effect on multiple protocorms development from seed drive protocorms after 12 weeks of culture.

BA at 2 mg/L in combination with IAA (2.0 and 1.0 mg/L) increased the shoot length (2.19 ± 0.01 cm and 2.33 ± 0.10 cm) but continuously released more phenols into the medium (Figure 6, **c**). KIN (1.0 mg/L) along with IAA (1.0 mg/L) was best (Figure 6, d) in producing multiple protocorms (22.40 ± 1.33) and more number of roots (1.60 ± 0.24). In addition, NAA at 0.5 mg/L and in combination with KIN at 2 mg/L produced better number of roots (1.60 ± 0.40 cm) and less number of multiple protocorms (1.27 ± 0.02). KIN (1.0 mg/L) + NAA (0.5 mg/L) was effective in increasing the root number (1.55 ± 0.17) and root length (0.99 ± 0.14 cm) (Table 3). In general phenol was released in the medium irrespective of the growth regulators. To prevent the phenolic exudation activated charcoal at 0.5 mg/L was added to the growth regulators free medium (Figure 6, g).

**Table 3**. Effect of combination of growth regulators on formation of multiple protocorms.

| Growth regulators (mg/L) | | | | Shoot length Mean ± S.E (cm) | No. of multiple protocorms/ explants Mean ± S.E | No. of roots Mean ± S.E | Root length Mean ± S.E (cm) |
|---|---|---|---|---|---|---|---|
| BAP | Kn | NAA | IAA | | | | |
| 0 | 0 | 0 | 0 | $1.22 \pm 0.05^{defghijk}$ | $1.00 \pm 0.00^{g}$ | $1.40 \pm 0.07^{ab}$ | **$1.02 \pm 0.04^{a}$** |
| 0.5 | - | 0.5 | - | $1.84 \pm 0.11^{b}$ | $2.40 \pm 0.60^{fg}$ | $1.30 \pm 0.34^{abc}$ | $0.49 \pm 0.16^{cde}$ |
| 0.5 | - | 1.0 | - | $1.41 \pm 0.23^{cde}$ | $1.00 \pm 0.00^{g}$ | $0.80 \pm 0.20^{abcdef}$ | $0.32 \pm 0.17^{defg}$ |
| 0.5 | - | 2.0 | - | $0.87 \pm 0.05^{klmno}$ | $2.40 \pm 0.51^{fg}$ | $0.40 \pm 0.24^{def}$ | $0.06 \pm 0.04^{g}$ |
| 1.0 | - | 0.5 | - | $1.37 \pm 0.68^{cdefg}$ | $1.60 \pm 0.60^{g}$ | $0.95 \pm 0.25^{abcde}$ | $0.24 \pm 0.07^{efg}$ |
| 1.0 | - | 1.0 | - | $0.85 \pm 0.10^{klmno}$ | $3.80 \pm 1.24^{fg}$ | $0.60 \pm 0.24^{bcdef}$ | $0.08 \pm 0.04^{g}$ |
| 1.0 | - | 2.0 | - | $0.54 \pm 0.07^{o}$ | $1.80 \pm 0.49^{fg}$ | $0.00 \pm 0.000^{f}$ | $0.00 \pm 0.000^{g}$ |
| 2.0 | - | 0.5 | - | $1.39 \pm 0.07^{cdef}$ | $2.00 \pm 0.63^{fg}$ | $0.30 \pm 0.20^{def}$ | $0.11 \pm 0.07^{g}$ |
| 2.0 | - | 1.0 | - | $0.88 \pm 0.08^{klmno}$ | $1.00 \pm 0.00^{g}$ | $0.80 \pm 0.20^{adcdef}$ | $0.17 \pm 0.05^{efg}$ |
| 2.0 | - | 2.0 | - | $0.90 \pm 0.13^{jklmno}$ | $1.00 \pm 0.00^{g}$ | $0.00 \pm 0.000^{f}$ | $0.00 \pm 0.000^{g}$ |
| 0.5 | - | - | 0.5 | $0.68 \pm 0.02^{mno}$ | $2.80 \pm 0.92^{fg}$ | $1.00 \pm 0.00^{abcde}$ | $0.24 \pm 0.02^{cde}$ |
| 0.5 | - | - | 1.0 | $1.14 \pm 0.08^{efghijklm}$ | $1.40 \pm 0.40^{g}$ | $1.00 \pm 0.00^{abcde}$ | $0.48 \pm 0.03^{cbe}$ |
| 0.5 | - | - | 2.0 | $1.37 \pm 0.12^{cdefg}$ | $1.00 \pm 0.00^{g}$ | $1.00 \pm 0.45^{abcde}$ | $0.32 \pm 0.13^{defg}$ |
| 1.0 | - | - | 0.5 | $0.94 \pm 0.06^{jklmn}$ | $3.00 \pm 0.95^{fg}$ | $0.20 \pm 0.20^{ef}$ | $0.06 \pm 0.06^{g}$ |
| 1.0 | - | - | 1.0 | $1.17 \pm 0.24^{defghijk}$ | $3.40 \pm 1.29^{fg}$ | $0.00 \pm 0.000^{f}$ | $0.00 \pm 0.000^{g}$ |
| 1.0 | - | - | 2.0 | $0.99 \pm 0.14^{hijklmn}$ | $2.00 \pm 0.63^{fg}$ | $0.00 \pm 0.000^{f}$ | $0.00 \pm 0.000^{g}$ |
| 2.0 | - | - | 0.5 | $1.71 \pm 0.11^{bc}$ | $1.00 \pm 0.00^{g}$ | $0.20 \pm 0.20^{ef}$ | $0.06 \pm 0.06^{g}$ |

**Table 3**. Continued.

| Growth regulators (mg/L) | | | | Shoot length Mean ± S.E (cm) | No. of multiple protocorms/ explants Mean ± S.E | No. of roots Mean ± S.E | Root length Mean ± S.E (cm) |
|---|---|---|---|---|---|---|---|
| BAP | Kn | NAA | IAA | | | | |
| 2.0 | - | - | 1.0 | **2.19 ± 0.01$^{a}$** | 8.60 ± 1.03$^{e}$ | 0.00 ± 0.000$^{f}$ | 0.00 ± 0.000$^{g}$ |
| 2.0 | - | - | 2.0 | **2.33 ± 0.10$^{a}$** | 2.00 ± 0.63$^{fg}$ | 0.00 ± 0.000$^{f}$ | 0.00 ± .000$^{g}$ |
| - | 0.5 | 0.5 | - | 0.94 ± 0.22$^{jklmn}$ | 2.80 ± 1.11$^{fg}$ | 1.10 ± 0.29$^{abcd}$ | 0.83 ± 0.31$^{ab}$ |
| - | 0.5 | 1.0 | - | 0.63 ± 0.03$^{no}$ | 11.80 ± 1.77$^{d}$ | 0.40 ± 0.24$^{def}$ | 0.16 ± 0.10$^{efg}$ |
| - | 0.5 | 2.0 | - | 0.73 ± 0.03$^{mno}$ | 1.40 ± 0.40$^{g}$ | 0.80 ± 0.37$^{abcdef}$ | 0.27 ± 0.11$^{efg}$ |
| - | 1.0 | 0.5 | - | 1.70 ± 0.10$^{bc}$ | 17.40 ± 1.63$^{b}$ | **1.55 ± 0.17$^{a}$** | **0.99 ± 0.14$^{a}$** |
| - | 1.0 | 1.0 | - | 0.65 ± 0.04$^{mno}$ | 14.40 ± 0.87$^{c}$ | 0.60 ± 0.24$^{bcdef}$ | 0.20 ± 0.09$^{efg}$ |
| - | 1.0 | 2.0 | - | 0.75 ± 0.07$^{mno}$ | 2.40 ± 0.60$^{fg}$ | 0.40 ± 0.24$^{def}$ | 0.28 ± 0.17$^{efg}$ |
| - | 2.0 | 0.5 | - | 1.27 ± 0.02$^{defghij}$ | 1.00 ± 0.00$^{g}$ | **1.60 ± 0.40$^{a}$** | 0.45 ± 0.06$^{cdef}$ |
| - | 2.0 | 1.0 | - | 1.03 ± 0.05$^{fghijklm}$ | 1.40 ± 0.40$^{g}$ | 1.40 ± 0.24$^{ab}$ | 0.48 ± 0.11$^{cdef}$ |
| - | 2.0 | 2.0 | - | 0.79 ± 0.08$^{lmno}$ | 1.20 ± 0.20$^{g}$ | 0.60 ± 0.24$^{bcdef}$ | 0.18 ± 0.09$^{efg}$ |
| - | 0.5 | - | 0.5 | 1.02 ± 0.01$^{gfijrklm}$ | 2.60 ± 1.03$^{fg}$ | 1.00 ± 0.00$^{abcde}$ | 0.65 ± 0.02$^{bcd}$ |
| - | 0.5 | - | 1.0 | 0.85 ± 0.14$^{klmno}$ | 4.60 ± 1.36$^{f}$ | 0.60 ± 0.24$^{bcdef}$ | 0.32 ± 0.13$^{defg}$ |
| - | 0.5 | - | 2.0 | 1.33 ± 0.07$^{defgh}$ | 4.60 ± 0.68$^{f}$ | 1.00 ± 0.45$^{abcde}$ | 0.48 ± 0.20$^{cde}$ |
| - | 1.0 | - | 0.5 | 0.94 ± 0.06$^{jklmn}$ | 2.60 ± 0.81$^{fg}$ | 0.20 ± 0.20$^{ef}$ | 0.06 ± 0.06$^{g}$ |
| - | 1.0 | - | 1.0 | 1.32 ± 0.20$^{defghi}$ | **22.40 ± 1.33$^{a}$** | **1.60 ± 0.24$^{a}$** | 0.68 ± 0.06$^{bc}$ |
| - | 1.0 | - | 2.0 | 1.53 ± 0.11$^{bcd}$ | 3.00 ± 0.84$^{fg}$ | 0.60 ± 0.40$^{bcdf}$ | 0.12 ± 0.07$^{fg}$ |
| - | 2.0 | - | 0.5 | 0.95 ± 0.10$^{ijklmn}$ | 3.20 ± 1.36$^{fg}$ | 0.00 ± 0.00$^{f}$ | 0.00 ± 0.00$^{g}$ |
| - | 2.0 | - | 1.0 | 1.43 ± 0.18$^{cde}$ | 2.60 ± 0.51$^{fg}$ | 1.10 ± 0.33$^{abcd}$ | 0.26 ± 0.08$^{efg}$ |
| - | 2.0 | - | 2.0 | 1.19 ± 0.08$^{defghijk}$ | 2.20 ± 0.73$^{fg}$ | 0.50 ± 0.32$^{cdef}$ | 0.09 ± 0.06$^{g}$ |

*****Mean ± Standard error followed by the common letter are not significant at the P ≤ 0.05% level by DMRT.

**Table 4**. Effect of organic additives on multiple protocorm induction in *T. spathulata*.

| Kn (mg/L) | Organic additives / 100 mL | | | No. of multiple protocorms / explant |
|---|---|---|---|---|
| | P (g) | T (mL) | CW (mL) | Mean ± S.E |
| - | 0.125 | - | - | $5.62 \pm 0.41^{cd}$ |
| - | 0.25 | - | - | $4.76 \pm 0.43^{cde}$ |
| - | 0.5 | - | - | **$14.64 \pm 0.31^{a}$** |
| - | - | 5 | - | $6.04 \pm 0.46^{c}$ |
| - | - | 10 | - | $7.92 \pm 0.68^{b}$ |
| - | - | 15 | - | $4.42 \pm 0.26^{de}$ |
| - | - | - | 5 | $6.02 \pm 0.51^{c}$ |
| - | - | - | 10 | $4.46 \pm 0.10^{de}$ |
| - | - | - | 15 | $4.06 \pm 0.20^{e}$ |
| 0.5 | 0.125 | - | - | **$16.84 \pm 0.63^{a}$** |
| 0.5 | 0.25 | - | - | $3.60 \pm 0.54^{d}$ |
| 0.5 | 0.5 | - | - | $3.66 \pm 0.49^{d}$ |
| 0.5 | - | 5 | - | $8.56 \pm 0.26^{c}$ |
| 0.5 | - | 10 | - | $8.94 \pm 0.46^{c}$ |
| 0.5 | - | 15 | - | $11.06 \pm 0.41^{b}$ |
| 0.5 | - | - | 5 | $8.13 \pm 0.31^{c}$ |
| 0.5 | - | - | 10 | $8.74 \pm 0.60^{c}$ |
| 0.5 | - | - | 15 | $8.02 \pm 0.47^{c}$ |

*****Mean ± Standard error followed by the common letter are not significant at the P ≤ 0.05% level by DMRT.

**Effect of organic additives**. To study the effect of organic additives on multiple protocorm induction (Figure 7, a) from seed derived protocorms, half strength MS medium was enriched with different concentrations of organic additives like peptone (P - 0.125, 0.25 and 0.5%), coconut water (CW - 5, 10 and 15%) and tomato juice (T - 5, 10 and 15%) with or without growth regulator (KIN - 0.5 mg/L). The results were recorded after 12 weeks of culture and presented in Table 4. The results indicated that all the concentrations of organic additives either individually or in combination with growth regulators (KIN - 0.5 mg/L) showed better response in increasing number of protocorm formation. Among the organic additives, peptone at 0.5% alone and 0.125% in combination with KIN (0.5 mg/L) gave best and early response in producing more number of multiple protocorms, ie., 14.64 $\pm$ 0.312 (Figure 7, b) and 16.84 $\pm$ 0.639 (Figure 7, c), respectively.

An increase in the concentration of peptone from 0.125 to 0.5 mg/L resulted in the release of more amount of phenol which finally led to death of the protocorms (Figure 7, f). Addition of tomato juice prevents the phenolic exudation from the protocorm into the medium and produced more number of multiple protocorms at 15% in growth regulator containing media (11.06 $\pm$ 0.41) (Figure 7, d). Coconut water produced less number of multiple protocorms in all treatments (Figure 7, e) but induce root formation in some explants.

**Rooting**

To induce rooting, the *in vitro* raised seedlings were transferred to 1/2 strength MS medium supplemented with IAA and NAA individually at three different concentrations ranging from 0.5-2.0 mg/L. Both NAA and IAA at 0.5 mg/L produced an average of 2.44 $\pm$ 0.224 and 2.43 $\pm$ 0.172 roots per shoot respectively and their root length was 1.46 $\pm$ 0.110 cm and 1.43 $\pm$ 0.086 cm, respectively. Figure 8 and 9 shows the formation of roots under the influence of auxin.

**Hardening**

The *in vitro* derived plantlets with well developed roots (Figure 7, g) were transferred to the potting media containing vermiculite (Figure 7, h). Plantlets were covered with a polyethylene bag and

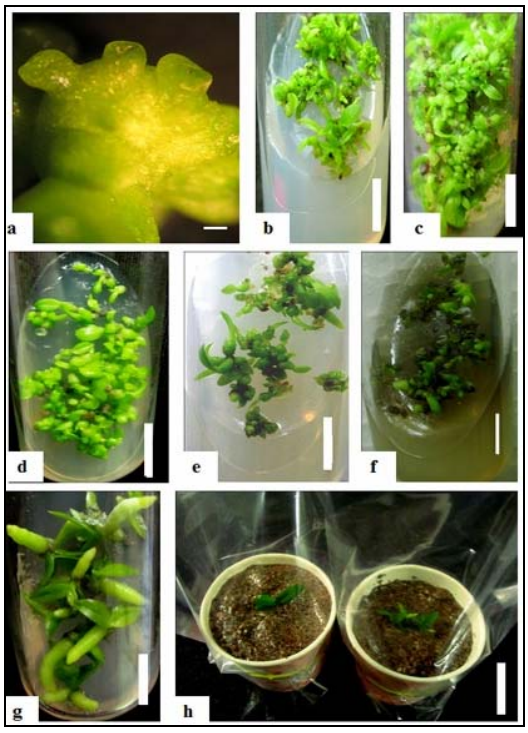


**Figure 7.** (a) Stereo microscopic view of multiple protocorms (Bar = 10 x 100 = 1000 µm). (b - e) Multiple protocorms developed on (b) 1/2 MS + peptone (0.5 mg/L) (Bar = 0.5 cm); (c) 1/2 MS + Kn (0.5 mg/L) + peptone (0.125 mg/L) (Bar = 0.5 cm); (d) 1/2 MS + Kn (0.5 mg/L) + tomato juice (15%) (Bar = 0.5 cm); (e) 1/2 MS + coconut water (15%) (Bar = 0.5 cm); (f) Phenols released in 1/2 MS + Kn (0.5 mg/L) + peptone (0.5 mg/L) (Bar = 0.25 cm); (h) Seedlings with well developed shoot and root (bar = 2.5 cm); (g) Hardening of *in vitro* raised plant (Bar = 2.5 cm).

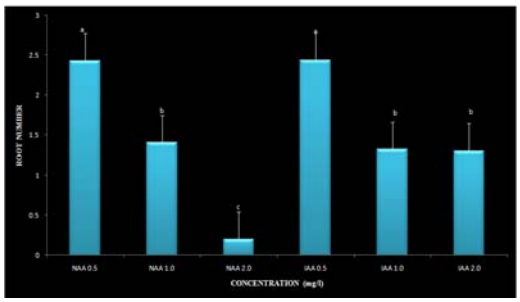


**Figure 8**. Effect of IAA ad NAA on root number.

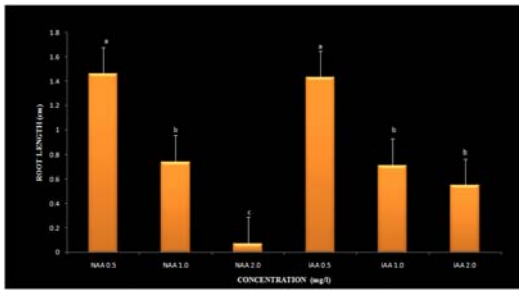


**Figure 9**. Effect of IAA and NAA on root length.

irrigated with sterile distilled water. The pots were maintained at 22 ± 2 ºC and the polyethylene cover was gradually loosened after 3 to 4 weeks, thus dropping the humidity (65%-70%). This procedure subsequently resulted in the *in vitro* hardening of the plantlets.

## Discussion

*T. spathulata* is an exquisite epiphytic orchid endemic to peninsular India and Sri Lanka. It is the only ex-*Vanda* with large golden yellow flowers. In the present investigation, an attempt was made for mass multiplication through green pod culture.

In *T. spathulata*, the percentage of seed viability was observed higher than the percentage of seed germination in the experiments by using tetrazolium staining. However, many studies on the Orchidaceae indicate that viability testing may not be a good indicator of germinability (Lauzer et al., 1994; Vujanovic et al., 2000; Johnson et al., 2007; Mahendran and Narmatha Bai, 2009). Because of this study, the viability of seeds estimated should be confirmed with germination tests.

In the present study, of the seven media tested, 1/2 strength MS medium showed significantly higher (92.73 ± 1.18%) percentage of seed germination

followed by 1/4 strength MS (90.70 ± 1.584%) after 8 weeks of culture initiation. Besides 1/2 strength MS medium, other five media also supported moderate germination (85.96 ± 6.06% in B5, 76.07 ± 2.41% in Mitra, 68.34 ± 3.92% in MS, 51.47 ± 4.20% in KCM and 48.83 ± 3.32% in LO). However, in $B_5$, M, LO and KCM germinated seeds failed to differentiate either onto protocorms or plantlets. The poor response of seed germination on $B_5$ media has also been reported for other orchids (Sharma et al., 1991; Dohling et al., 2008) despite this media being suitable for *Dendrobium chrysanthum* (Hajong et al., 2010).

Cytokinin treatment (BA and KIN) results in more number of multiple protocorm formations in *Dendrobium*, *Oncidium* and *Cattleya* (Saiprasad et al., 2002). Both BA and KIN were used in *Satyrium nepalense* (Mahendran and Narmatha Bai, 2009), *Vanda testacea* (Kaur and Bhutani, 2009) and *Dendrobium nobile* (Sana et al., 2011) to induce multiple shoot and BA induced more number of multiple shoots than KIN. This is in disagreement with the present experiment, since KIN was more effective for inducing maximum number of multiple protocorms (12.00 ± 0.27). These correlate with the reports of Fonnesbech (1972) in *Cymbidium,* Chen et al. (2002) in *Epidendrum radicans* and Dhiman et al. (2013) in *Habenaria edgeworthii*. The cultured protocorms of *T. spathulata* produced large number of shoot buds and exhibited minimum shoot length which was due to sharing of nutrition by many lateral shoots (Yakimova et al., 2000), in this study similar result was observed.

BA employed in the present study to increase the shoot length (1.74 ± 0.21) in *Taprobanea spathulata*. Similar observation were made by Pierik and Steegmans (1972) in *Cattleya aurantice*, Pathak et al. (2001) in *Cymbidium pendulum*, Luo et al. (2006) in *Dendrobium densiflorum*, Bhadra and Hossain (2003) in *Geodorum densiflorum*, Pant and Hapa (2012) in *Dendrobium primulinum*, and Arenmongla and Deb (2013) in *Malaxis acuminata*. BA at higher concentrations did not produce root. Similar results were observed in *Cypripedium candidum* (De-Pauw et al., 1995) and *Dendrobium malones* (Anjum et al., 2006). In this study 1/2 strength MS media devoid of growth regulators increased the root length as reported in *Habenaria edgeworthii* (Giri et al., 2012).

In the present investigation multiple protocorm formation was significantly high in medium supplemented with KIN and IAA which is in agreement with Mathews and Rao (1980) in *Vanda* hybrids and Hadley (1970) in *Dactylorhiza purpurella* where growth rate had been enhanced. BA in combination with IAA increased shoot length of *Vanda coerulea* (Manners et al., 2010). Similar results were observed in *T. spathulata*.

Auxin was the first plant growth regulator added to orchid seed culture to enhance seedling growth (Arditti, 1979; Nasiruddin et al., 2003). Under *in vitro* conditions, supplementation of exogenous hormone (auxins) to the medium enhances rooting. In *T. spathulata,* both NAA and IAA (0.5 mg/L) were effective in inducing better root system and seedling development.

The stimulatory effect of IAA has been reported in *Vanda* (Rao and Avadhani, 1963), *C. giganteum* (Hossain et al., 2010), *Dendrobium aphyllum* (Hossain et al., 2012), *Dendrobium primulinum* (Pant and Thapa, 2012) and *Orchis coriophora* (Bektaş et al., 2013).

The beneficial effect of NAA on leaf and root development was reported by Chung and Chun (1983). In *T. spathulata,* the seedlings were produced in NAA at lower concentration which was also reported by Mitra (1976). NAA induced root formation in *Vanda tessellata* (Rahman et al., 2009), *Eria bambusifolia* (Basker and Narmatha Bai, 2010), *Cymbidium aloifolium* (Potshangbam and Nirmala, 2011), *Malaxis acuminata* (Arenmongla and Deb, 2012). Auxins inhibited the growth of shoots and roots in *Paphiopedilum spicerianum* (Kano, 1965), and *Dendrobium chrysanthamum* (Miyazaki and Nagamatsu, 1965).

Organic additives were added to the culture medium to promote *in vitro* regeneration, growth and proliferation of orchids (Ichihashi and Islam, 1999; Chai et al., 2002; Chen and Chang, 2002; Islam et al., 2003; Rahman et al., 2004; George et al., 2008; Ng and Saleh, 2011). For the proliferation of seed derived protocorms of *T. spathulata,* coconut water, peptone and tomato juice were used alone or in combination with growth regulator (KIN 0.5mg/L).

Tomato extract contains 93.1% moisture, 4.89% carbohydrate (glucose and fructose), protein, lycopene, vitamins, minerals, low protein, fat, organic acids and strong antioxidants. (Abdel-Rahman and Abdel-Hamd, 1982). The enhanced effect of tomato homogenate on seed germination as well as growth of PLBs was reported in *Geodorum densiflorum* (Muthukrishnan et al., 2013).

Coconut water was commonly used in the tissue culture. Growth promotory nature of CW is related to its ability of inducing cell divisions in non-dividing cells hence promoting early protocorm differentiation (Intuwong and Sagawa, 1973). Addition of CW (15%) to the basal medium increased growth of the cultures and the shoots vigorously rooted in epiphytic orchids (McIntyre et al., 1974).

In *T. spathulata,* the addition of organic additives either individually or in combination with KIN (0.5) induced more number of PLBs production. A combination of KIN and peptone was found to be most effective as reported in *Dendrobium moschatum* (Singh et al., 2013), but in *Paphiopedilum* the same combination induced callus formation (Chyuam, and Norihan, 2011). Organic additives along with growth regulators proved to be beneficial for induction of more number of PLBs in *T. spathulata* which was due to synergetic effect of growth regulator and any of the component(s) present in the organic additives. Further studies are required to determine the factor(s) responsible for the promotory effect.

Excessive phenolic production was possibly due to increased polyphenol oxidase and catalase activity triggered by certain cultural conditions (Harvais, 1982; Basker and Narmatha Bai, 2010; Mahendran et al., 2013). Phenolic exudations from the seedlings were observed in all the treatments. Browning of the medium, followed by seedling death and blackening of the leaf tips was common in all the cultures, which affected the survival rate of the plantlets. So, activated charcoal has been used to prevent excessive phenolic exudates, promote growth and maintenance of cultures. This beneficial effect have been reported in *Zygopetalum intermedium* (Ram Pal et al., 2013) and *Coelogyne cristata* (Sharma, 2013).

The success of *in vitro* propagation lies in establishment of plant in the soil (Saxena and Dhawan, 1999; Deb and Imchen, 2010). For *ex vitro* establishment of *T. spathulata,* vermiculite was used and proved to be effective for hardening as reported in *Satyrium nepalense* (Mahendran and Narmatha Bai, 2009).

## Conclusion

In conclusion, a simple, efficient protocol for mass propagation of *T. spathulata* an medicinally important epiphytic orchid from green capsules has been established. Since the flowers of this species are conspicuous and showy, this can be exploited for commercial production and for hybridization program for the production of new hybrids. Tissue culturing and the *in vitro* propagation of *T. spathulata* explants could be possible method for the large scale commercial production of biologically active compounds.

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## Conflict of interest statement

Authors declare that they have no conflict of interests.

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