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RESEARCH ARTICLE

Optimization of culture media for micropropagation of *Phaius tankervilleae* (Banks) Blume, a terrestrial orchid native to Arunachal Pradesh

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Abstract

Phaius tankervilleae (Banks) Blume is a terrestrial sympodial orchid native to Arunachal Pradesh. It thrives in dense moist tropical forests at altitudes ranging from 1000 to 2000 meters. The orchid holds economic significance in floriculture, natural dye production, and traditional medicine. However, it faces threats to its survival due to various anthropogenic activities, and low seed germination (only 5%) in natural conditions. In the present study, we aimed to formulate a suitable medium for the micropropagation of this orchid. Our findings revealed that for in vitro asymbiotic seed germination, protocorm formation, and seedling development, both full strength and ½ strength Murashige and Skoog (MS) were better basal media in comparison to ¼ strength MS, Knudson C, Vacin and Went, and Heller medium, although embryo swelling occurred after 7 weeks of culture (WOC) on all of them. Among the organic additives screened for media supplementation, peptone emerged as a superior option compared to coconut water or yeast extract on whose supplementation at 2 g/l into both full MS and ½ MS media resulted in early embryo swelling (4 WOC), along with high seed germination (51.5%) and protocorm formation (44.7%) after 10 WOC.

Keywords: Orchid cultivation; Tissue Culture; Asymbiotic seed germination; Protocorm formation; Media formulation; Peptone supplementation

1. Introduction

Orchids are well known for their splendid, long lasting flowers which are in high demand both in international and national market. Due to indiscriminate exploitation and habitat destruction, the entire Orchidaceae family is under a serious threat and have been put under restricted list of trade under Appendix-II of the Convention on International Trade in Endangered Species of Fauna and Flora (CITES, 2021). Although orchids produce numerous seeds but by virtue of being non-endospermic, strongly hydrophobic due to thick seed coat, and accumulation of inhibitory substances like abscisic acid within mature seeds, their rate of germination is merely 5%, and for their germination, growth and development, many of the orchid species rely upon mycorrhizal fungi (Arditti and Ghani, 2000; Li et al., 2021; Yeung, 2017). As orchid plants are slow growing and produce only a limited number of vegetative offspring, therefore, micropropagation through asymbiotic seed germination of immature seeds has been recommended for their large scale production (Arditti et al., 1982; Yeung, 2017). Nevertheless, during micropropagation most of the orchid species through explants other than immature seeds produces recalcitrant calli that fail to develop into seedlings. It is also well established that the success rate of in vitro micropropagation through asymbiotic culture depends upon the composition of the culture media as every orchid species requires specific media formulation. Various workers have explored different growth media and organic additives (e.g. casein hydrolysate, chitosan, coconut water, fruit based homogenates, peptone, yeast extract) that serve as a natural source of amino acids, vitamins, minerals, growth regulators, and nitrogen for micropropagation of different orchids (Utami and Hariyanto, 2020).

For the present work, we selected *Phaius tankervilleae* (Banks) Blume, a rare, terrestrial, sympodial orchid commonly known as the "Nun's-hood orchid" (Kanwal, 2014; Photo plate 1A). It is distributed in Kameng, Lohit, Lower Dibang Valley, Siang, and Tirap districts of Arunachal Pradesh, and thrives in shady habitats inside tropical and sub-tropical forests at 1000-2000 msl and flowers splendidly during April to June (Chowdhery, 1998; Kanwal, 2014). Economically it is important in floriculture (Kanwal, 2014), traditional medicine (Medhi and Chakrabarti, 2009) and natural dyes production (Mahanta and Tiwari, 2005).

Only a few but very preliminary studies on micropropagation of P. tankervilleae through asymbiotic seed culture are available (Pant et al., 2011; Thockchom et al., 2017; Vimal et al., 2018). However, none of these have explored various formulations using different basal media and organic amendments. Therefore, in the present work, various media formulation involving different basal media and organic amendments were employed to study their beneficial role in asymbiotic culture of immature seeds, protocorm formation and seedling development.

2. Materials and methods

2.1 Seed source and sterilization

A healthy 5-6 months old capsule of *P. tankervilleae* (Photo plate 1B) was collected from the Orchidarium of the department. It was washed under running tap water, and dirt on the surface was removed using a soft brush and a few drops of liquid detergent (Extran MA 02 neutral, Merck). Surface sterilization of the capsule was performed for 15 minutes under laminar airflow chamber using 1% Sodium hypochlorite mixed with 4-5 drops of Tween 20, and thereafter it was thoroughly washed with sterile double-distilled water until the soapiness was removed. After that, it was dipped in 70% ethyl alcohol for 5-10 sec. and subjected to quick flame sterilization.

2.2 Preparation of growth media, culture conditions and data recording

Growth media for *in vitro* asymbiotic seed culture was prepared by mixing Sucrose (30 g/l) and agar (8 g/l) to the basal medium as the carbon source and gelling agent respectively. The pH of the culture media was adjusted to 5.8±0.05 using 1N NaOH or 1N HCl. The culture media were then autoclaved at 121°C, 15 psi for 20 minutes and then left for two days for cooling and ensuring contaminant-free media. Immature seeds scooped out with the help of a spatula from the sterilized capsule after making longitudinal or transverse cut using a sterile surgical blade were inoculated on the growth medium

in sterile test tubes. After inoculation, all cultures were kept in an incubation room at 23±2°C under fluorescence light for a 16/8 hours light/dark photoperiod.

Various developmental stages of inoculated seeds were observed under the Stereomicroscope (Carl Zeiss, GmbH) and recorded as per Yamazaki and Miyoshi (2006; Table 1).

Table 1: Developmental stages in the germination of *P. tankervilleae* (modified from Yamazaki and Miyoshi, 2006)

Stage	Description
0	No growth of embryo occurs (No germination; Photo plate 1C)
1	Swelling of seed (Pre-germination)
2	Embryo emerging out by rapturing the testa (Germination)
3	Hairy protocorm
4	First leaf formation
5	Second leaf formation, root primordial and further development

2.3 Media screening for in vitro asymbiotic seed culture

Six different basal media, namely Full strength Murashige and Skoog (MS), ½ MS, ¼ MS, Knudson C (KC), Vacin and Went (VW), and Heller media (HM) were used for seed germination, protocorm formation, and seedling development. For each treatment, 24 replicates were kept (n=24). Cultures were observed weekly for 17 weeks and initial days of seed germination and protocorm formation were recorded. The percentage of seed germination, protocorm formation, and seedling development was calculated by dividing the number of germinated seeds, protocorms, and seedlings by the total number of inoculated seeds.

2.4 Effect of dark pre-treatment on seed germination and protocorm formation

On the basis of the results of previous experiment, full strength MS and ½ MS were adjudged as suitable basal media for subsequent experiments. The effect of dark pre-treatment on seed germination and protocorm formation was investigated by subjecting seed inoculated test tubes to 5, 10, and 15 days of dark pre-treatment before transferring them to a 16/8 hours light/dark cycle. For each treatment, 24 replicates were kept, and the cultures were observed weekly for 10 weeks. The percentage of seed germination and protocorm formation were calculated as mentioned earlier.

2.5 Effect of organic amendment on seed germination and protocorm formation

Full strength MS and ½ MS basal media were supplemented with different organic amendments at varying concentrations {Coconut water (CW) at 0, 50, 100, 150, 200 ml/l; Peptone at 0, 1, 2, 3 g/l; Yeast extract (YE) at 0, 2, 4, 6 g/l} to find out their effect on seed germination and protocorm formation. For each treatment, 15 replicates were kept, and cultures were observed weekly for 10 weeks. The percentage of seed germination and protocorm formation was calculated as mentioned earlier.

2.6 Experimental design and data analysis

The present study was carried out in a completely randomized design (CRD). Data were analysed using ANOVA (Microsoft Excel) and means were compared using the Least Significant Difference (LSD) at p=0.05.

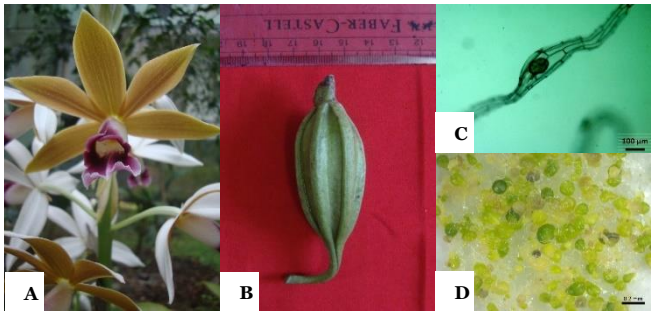

Photo plate 1: (**A**) A full bloomed flower of *P. tankervilleae*; (**B**) A capsule (5-6 months old); (**C**) A seed (Stage 0); (**D**) Protocorm formation on MS medium supplemented with Peptone @ 2 g/l. after 10 weeks of culture.

3. Results

3.1 Media screening for in vitro asymbiotic seed culture

After 7 weeks of culture (WOC), inoculated seeds of *P. tankervilleae* showed embryo swelling (Stage 1), and germinated subsequently (Stage 2) on every basal media used in the study (MS, ½ MS, ¼ MS, HM, KC and VW). After 17 WOC, MS and ½ MS media supported maximum seed germination (35.4–38.5%) and protocorm formation (14.4–16.1%) followed by ¼ MS, VW and KC (Fig. 1). Seedling development could happen only on MS and ½ MS media. On the other hand, on KC and VW, protocorms underwent necrosis just after a few weeks. On HM, though the seed testa ruptured but the embryo (Stage 2) remained colourless (no chlorophyll) and eventually died.

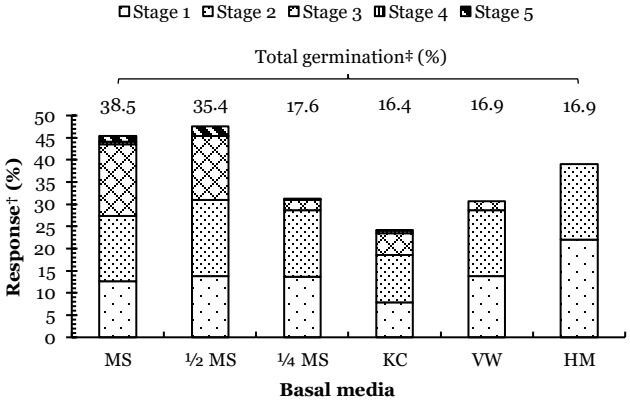

Figure 1. Effect of basal media on asymbiotic seed culture after 17 WOC (Mean±SEM; N=24; p<0.05 for Stages 1, 3, 5 and Total germination; p>0.05 for Stages 2 and 4). †Sum total of seeds attaining Stage 1 to 5; ‡Sum total of seeds attaining Stage 2 to 5.

3.2 Effect of dark pre-treatment on seed germination and protocorm formation

The seed germination (Stage 2) and protocorm formation (Stage 3) were recorded on the two best performing growth media (MS or ½ MS) after 10 weeks of culture (WOC) that was counted from the day of completion of dark pre-treatment period (0-15 days). Five-day period of dark pre-treatment supported maximum seed germination (Figure 2). Further, there was no difference in terms of growth media irrespective of treatment period. However, enhanced seed germination due to dark pre-treatment was coupled with a reduction in the number of protocorms formed, and an increase in the duration of treatment had a further negative effect (Figure 3).

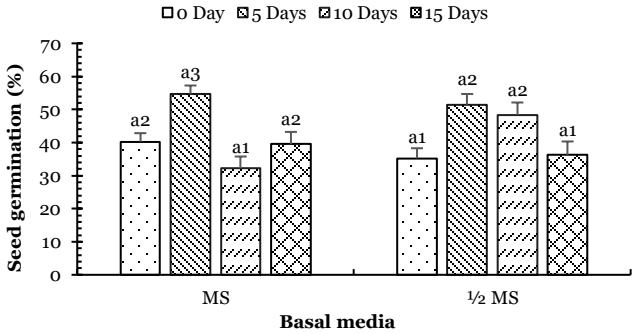

Figure 2. Effect of dark pre-treatment (0-15 days) on seed germination after 10 WOC (Mean±SEM; N=24; p>0.05 for Basal media; p<0.05 for Dark pre-treatment, and Basal media×Dark pre-treatment. Means having the same letter and numeral are significantly not different for media and days of pre-treatment, respectively).

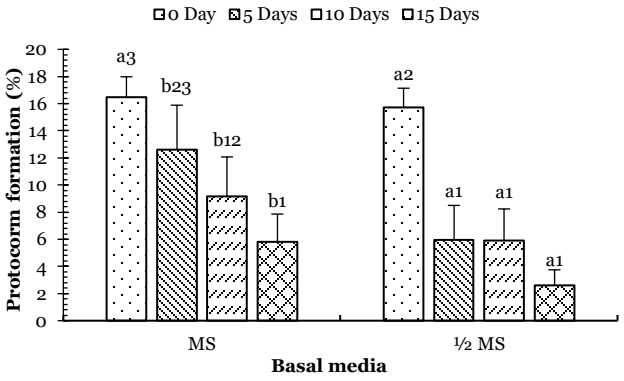

Figure 3. Effect of dark pre-treatment on protocorm formation after 10 WOC (Mean±SEM; N=24; p<0.05 for Basal media and Dark pre-treatment; p>0.05 for Basal media×Dark pre-treatment. Means having the same letter and numeral are significantly not different for media and days of pre-treatment, respectively).

3.3 *Effect of organic amendment on seed germination and protocorm formation*

Different organic amendments at various concentrations (CW at 0, 50, 100, 150, 200 ml/l; Peptone at 0, 1.0, 2.0, 3.0 g/l; YE at 0, 2.0, 4.0, 6.0 g/l) were studied for their effects on seed germination, and protocorm formation after 10 WOC.

Seeds germination (Stage 2) occurred quickly as evident by embryo swelling beginning after 4 WOC on both the basal media supplemented with any one of the organic amendments. In control treatments, it happened after 7 WOC (data not shown).

Out of the three organic amendments tested, Peptone and YE but not CW appeared suitable in inducing a higher rate of seed germination. Addition of CW to any of the basal media did not enhance seed germination significantly (Figure 4). Nevertheless, the seed germination was always higher on MS medium. Among all the applied concentrations, addition of CW @150 ml/l to both the media resulted in a little more seed germination (Figure 4). Conversely, addition of Peptone and YE significantly enhanced seed germination on both the media whose were on par in performance with each other (Figure 5 and 6). Further, both Peptone and YE performed much better than the CW. A significantly higher rate of seed germination was obtained on addition of Peptone and YE @ 1g/l and 4g/l respectively on both the media (Figure 5 and 6).

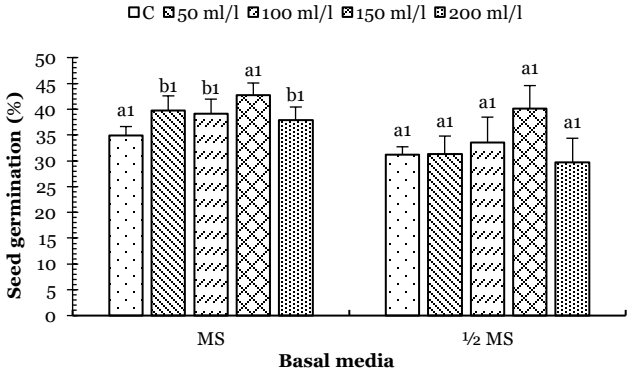

Figure 4. Effect of different concentration of Coconut water on seed germination after 10 WOC (Mean±SEM; N=15; p<0.05 for Basal media; p>0.05 for CW conc., and Basal media×CW conc. Means having the same letter and numeral are significantly not different for media and conc. of CW, respectively).

Among the three organic amendments, it was only Peptone that significantly enhanced protocorm formation (Fig. 7, 8 & 9). Its best concentration was 2g/l and 3g/l providing 44.7% and 52.2% protocorm formation (Photo plate 1D) on MS and ½ MS respectively (Figure 8). Addition CW and YE drastically reduced protocorm formation almost equally on both the media, and their negative effect were more pronounced with further increasing the dose (Figure 7 & 9). Nevertheless, the negative effect was equal in MS and ½ MS for CW whereas lesser in ½ MS than MS for YE.

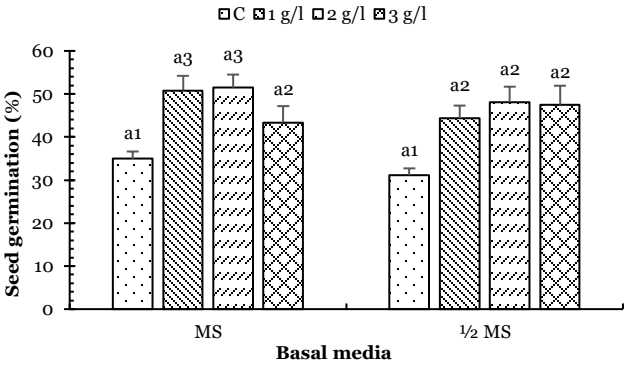

Figure 5. Effect of different concentration of Peptone on seed germination after 10 WOC (Mean±SEM; N=15; p>0.05 for Basal media, and Basal media×Peptone conc.; p<0.05 for Peptone conc. Means having the same letter and numeral are significantly not different for media and conc. of Peptone, respectively).

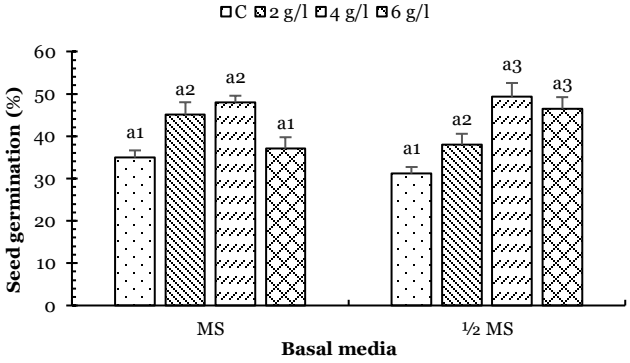

Figure 6. Effect of different concentration of Yeast extract on seed germination after 10 WOC (Mean±SEM; N=15; p>0.05 for Basal media; p<0.05 for YE conc., and Basal media×YE conc. Means having the same letter and numeral are significantly not different for media and conc. of YE, respectively).

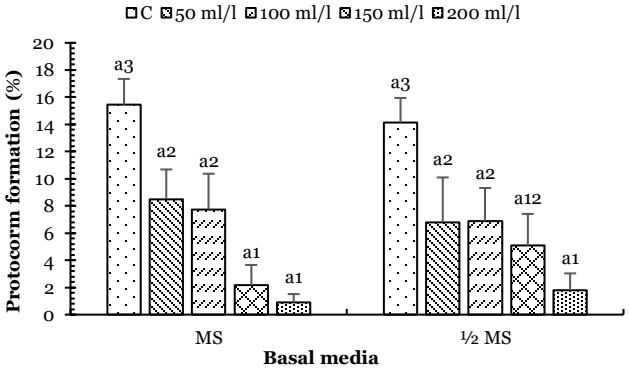

Figure 7. Effect of different concentration of Coconut water on protocorm formation after 10 WOC (Mean±SEM; N=15; p>0.05 for Basal media, and Basal media×CW conc.; p<0.05 for CW conc. Means having the same letter and numeral are significantly not different for media and conc. of CW, respectively).

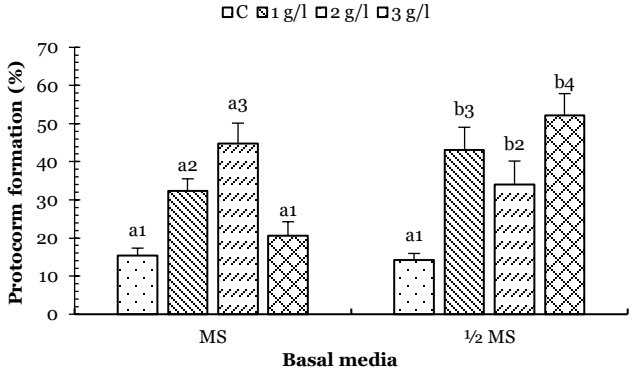

Figure 8. Effect of different concentration of Peptone on protocorm formation after 10 WOC (Mean±SEM; N=15; p<0.05 for Basal media, Peptone conc., and Basal media×Peptone conc. Means having the same letter and numeral are significantly not different for media and conc. of Peptone, respectively).

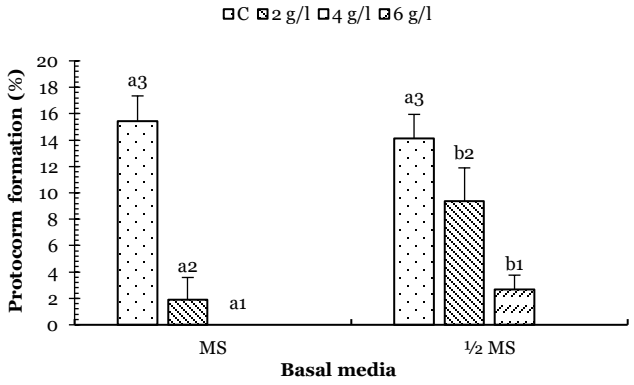

Figure 9. Effect of different concentration of Yeast extract on protocorm formation after 10 WOC (Mean±SEM; N=15; p<0.05 for Basal media, YE conc., and Basal media×YE conc. Means having the same letter and numeral are significantly not different for media and conc. of YE, respectively).

4. Discussion

4.1 Media screening for in vitro asymbiotic seed culture

Asymbiotic germination of orchid seeds on synthetic media is influenced by the type of media, and the salt concentration of the media. As the response of orchid is often media specific, selection of suitable medium is one of the crucial factor affecting the success rate for orchid culture. The present study demonstrates that both MS and ½ MS were comparatively better basal media than ¼ MS, KC, VW and HM for seed germination and protocorm formation in *P. tankervilleae*. The high seed germination and subsequent protocorm formation in MS and ½ MS media could be attributed to the ratio of ammonium and nitrate present in the medium (Park and Yeung, 2018), which serves as the primary nitrogen source for plant growth (Howitt and Udvardi, 2000). According to Atia et al. (2009), $NO3^-$ as nitrogen source enhances seed germination. Paul et al. (2012) and Mohanty et al. (2012) reported similar results for seed germination in *Dendrobium hookerianum* and *Cymbidium mastersii* respectively. The whitish embryo germination observed in HM might be due to its lower iron content compared to other tested media (Park and Yeung, 2018). Iron sufficiency in the growth medium is essential for the synthesis of chlorophyll and other iron-containing compounds like porphyrins or hemes otherwise its deficiency can lead to chlorosis in higher plants (Pushnik et al., 1984). In the present study, though protocorm formation happened in KC and VW, it gradually showed symptoms of necrosis, and ultimately died. A high mortality rate of protocorms in KC and VW could be attributed to the lack of essential vitamins and amino acids necessary for plant growth and development (Saad and Elshahed, 2012). A similar outcome was reported for *Cattleya* species, where protocorms exhibited necrosis after 63 days in KC medium (Hosomi et al., 2012).

4.2 Effect of dark pre-treatment on seed germination and protocorm formation

Photoperiod is a crucial factor influencing seed germination in orchids (Oliva and Arditti, 1984). The widely accepted notion that epiphytic orchids require light while terrestrial orchids require darkness for seed germination is based on natural conditions (Zeng et al., 2012). However, the germination responses to photoperiod are often species-specific, irrespective of growth habit (Kauth et al., 2008). In this study, a five-day dark pre-treatment significantly enhanced seed germination, whereas prolonged darkness decreased germination, suggesting that a five-day dark pre-treatment is optimal for seed germination in *P. tankervilleae*. Zeng et al. (2012) reported that a 45-day dark pre-treatment enhanced seed germination in *Paphiopedilum wardii*. While dark pre-treatment has been shown to have some effect on terrestrial orchid seed germination, the specific role of dark pre-treatment remains unclear. It might be an adaptive mechanism of terrestrial orchids, as seeds are typically buried in soil under dense forests after dispersal, receiving minimal light (Kauth et al., 2008). However, in this study, protocorm formation was poor under dark pre-treatment conditions. Zeng et al. (2012) also reported death of seedlings in media subjected to extended dark pre-treatment, suggesting for essentiality of light during seedling development. Nevertheless, leaf formation in 90% of protocorms under complete darkness is also reported in *Habenaria macroceratitis* (Stewart and Kane, 2006).

4.3 Effect of organic amendment on seed germination and protocorm formation

Organic amendments provide a natural source of carbohydrates, inorganic ions, amino acids, vitamins, nitrogen, and phytohormones, promoting growth and morphogenesis in orchid seed cultures (Nabieva, 2021). In the present study, amendment of MS and ½ MS with Coconut water (CW), Peptone, and Yeast extract (YE) shortened the period of seed germination in *P. tankervilleae* by approximately three weeks. Similar results have been reported in *Epidendrum ibaguense* (Hossain, 2008) and *Vanda dearei* (Jualang et al., 2014). Our study found just a little enhancement in the seed germination rate on addition of CW, however, the effect was not significant. Nevertheless, it caused a substantial decrease in protocorm formation. Other studies reports about its beneficial effect towards seed germination and growth (Huh et al., 2016; Piri et al., 2013; Zeng et al., 2012) ascribing to the presence of amino acids, vitamins, sugar, and plant growth regulators such as cytokinin (Laurain et al., 1993), and various inorganic ions (Raghavan, 1977). The addition of YE also enhanced the seed germination but with a substantial decrease in protocorm formation. It also resulted in protocorm necrosis. Jualang et al. (2014) also reported similar results in *Vanda dearei*.

Among three organic supplements, only Peptone could provide enhanced seed germination as well protocorm formation on both MS and ½ MS media. Addition of Peptone @ of 0.5 g/l, 1.0 g/l and 2.0 g/l is reported to promote seed germination in *Paphiopedilum* and *Vanda* species (Curtis, 1947), *Renanthera imschootiana* (Wu et al., 2014) and *Spathoglottis plicata* (Hossain and Dey, 2013) respectively. Buyun et al. (2004) and Utami et al. (2017) also observed similar results in *Dendrobium parishii* and *D. lasianthera*. As Peptone contains high levels of amino acids, vitamins, and nitrogen, therefore, it may enhance seed germination in orchids (Oliva and Arditti, 1984; George et al., 2007; Nhut et al., 2008; Dutra et al., 2008).

5. Conclusion

This study reports suitable basal media for seed germination, protocorm formation and seedling development. Additionally, the study suggest that dark pre-treatment of inoculated seeds may promote seed germination but inhibits protocorm formation. Media supplementation with Peptone under proper artificial illumination promotes seed germination as well as protocorm formation.

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Author(s) contribution

Tadu Yaniya: Formulated, designed and executed the research; Rajiv Kumar Singh: Mobilized funds, supervised the work, performed statistical analyses, and evaluated the manuscript. The authors have extensively reviewed and endorsed the final draft of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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