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PEPTONE AND TOMATO EXTRACT INDUCED EARLY STAGE OF EMBRYO DEVELOPMENT OF *Dendrobium Phalaenopsis* ORCHID

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ABSTRACT

Germination and growth of orchid seeds can be accelerated by the addition of organic supplement and plant extract in culture medium. The objective of this study was to determine the effect of peptone and tomato extract on early stage of embryo development of Dendrobium phalaenopsis orchids. Orchid seeds were sown on NP and VW medium with addition of 10% of CW (NPCW and VWCW). Five weeks after seed germination, about 58.03% of seed germination was observed on VWCW medium, and was only 37.45% seed germination on NPCW. After 8 weeks on VWCW medium with addition of 100 mlL^{-1} Tomato extract and 2 grL^{-1} peptone (termed as VWCWTP medium), 94.42% seeds were germinated into plantlet, but only 67.30% of seeds were germinated on VWCW. To get optimal growth and development of D. phalaenopsis orchids embryos in the in vitro condition, supplement of 100 mlL^{-1} coconut water, 100 mlL^{-1} tomato extract and 2 mgL^{-1} peptone into VW basic medium is required.

1. Introduction

Dendrobium phalaenopsis is an endemic orchid from Larat Island in the eastern Indonesia at Maluku region (Rahardi, 2006). This orchid is important germplasm of the country as a nation competitiveness because this orchid produce very beautiful flower with purple color (Charles & Baker, 1996), and round shape similar to *Phalaenopsis* (Anonim, 2003), so that this flower is very often used as parental to create potential orchid hybrid (Charles & Baker, 1996). Recently, the population of this orchid in its habitat was extremely decrease because of natural disaster, over-collection for commercial use or people do not know how to take care and to maintain the growth and condition similar to its the original habitat. To solve this problem, *in vitro* seed germination and micropropagation will be powerful as biotechnological approach.

It is well known, that the size of orchid seed is very small about 0.09 to 1.2 mm (width) and 0.25 to 1.2 mm (length). There are about 4 million seeds per capsule of orchids pod (Arditti, 1967) but lacks of endosperm (Shekarriz *et al*. 2014). Do to orchid seeds do not have endosperm, they are difficult to germinate. *In vitro* culture techniques is needed to germinate the seeds of orchids. Medium is one of the factors that influence germination of orchid seed (Semiarti *et al*., 2011). Component of medium for *in vitro* cultivation of orchid seed consists of macronutrients, micronutrients, vitamins, amino acids, myo-inositol and organic supplements Macronutrients substances needed in relatively large amounts. They include calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K), and sulfur (S) (George & Sherrington, 1984). Among the macronutrients, N has the greatest impact on plant growth (da Silva, 2013).

Germination of the orchid seed can be increased by the addition of organic supplements and plant extracts. Many different organic additives included coconut water, banana pulp, peptone and tomato extract (Shekarriz *et al.,* 2014). Coconut water (CW) is the liquid endosperm of green coconuts (*Cocos nucifera*) (Arditti 2008). Coconut water contains a number of amino acids, such as organic acids, nucleic acids,

several vitamins, sugars and sugar alcohols, plant hormones (auxins, cytokinins), minerals and other unidentified substances, responsible for promoting orchid seed germination and the growth of plantlet (Shekarriz *et al.,* 2014). Tomatoes contain sugar and antioxidants including vitamin C that can stimulate seed germination and the growth of protocorm (Semiarti *et al.,* 2010). Peptone contains amino acids and vitamins that also can stimulate the growth of protocorm (Oliva and Arditti, 1984). According to Butcher and Marlow (1986) and Suryowinoto (1996), a particular medium for the cultivation of orchid seeds is Knudson C (1946), Vacin and Went (1949) or Murashige and Skoog (1962).

The objective of this study was to determine the effect of macronutrients, particularly peptone (amino acid in various medium) on embryogenesis of *D. phalaenopsis* orchids.

2. Materials and Methods

2.1. Plant Materials and In Vitro Culture

Plant materials used for this study was 2.5 months old after self-pollination of the wild orchid *D. phalaenopsis* fruit/capsule, where the color is still green (Figure 1). Before the seeds were planted on culture media, the capsule was surface sterilized by dipping it in alcohol and then was burned a few seconds with a Bunsen flame. This work was repeated 3 times. The sterilized orchid capsule was opened with a sterile surgical blade, and the orchid seeds were sown on New Phalaenopsis (NP) and Vacin and Went (VW) medium, with addition of 100 mlL^{-1} coconut water, referred to NPCW and VWCWmedium. Based on the growing speed of orchid seeds on medium, both medium were compared to choose the better medium.The chosen better medium was added with 100 mlL^{-1} tomato extract and 2 mgL^{-1} peptone. The cultures were continously maintained at 25°C with continues white light (1000 lux) during this experiment.

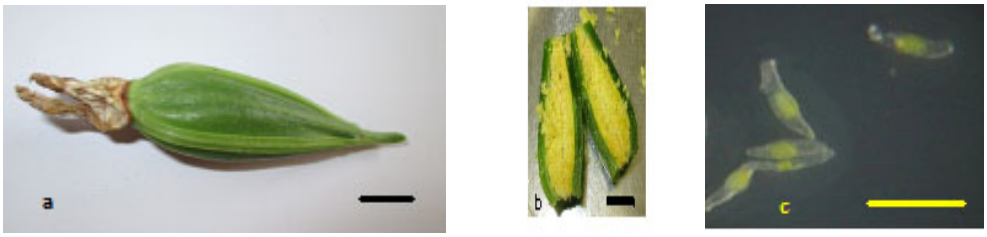

Figure 1. a. *D. phalaenopsis* orchid capsule, b. orchid capsule was split longitudinally, c. orchid seeds. Bar a & b: 1 cm, Bar in c: 0.5 mm

The developing orchid's embryo (protocorm) grew into plantlet. According to Semiarti *et al*. (2010), the embryo development in orchids were classified into 6 phases, namely phase 1: embryo grew into an ovoid-shape, phase 2: seed coat was cracked and embryo grew to be an ovoid- shape outside the seed coat (testa), phase 3: the shape of embryo became spherical and separated from the seed coat, this developing embryo was called protocorm; phase 4: Shoot apical meristem (SAM) will be formed at the upper part of protocorm, phase 5: protocorm form leaf primodium, and phase 6: protocorm form the first leaf. The observations were made every week. The percentage of protocorms and the length and width of protocorms were measured at 8 weeks after seed plantation (WASP) at the media with the addition of peptone and tomato extract.

2.2. Histological Analysis

For histological studies on the developmental process from embryo into protocorm, seeds and protocorms were fixed in a mixture solution of formaldehyde: acetic acid : 70% ethanol (1:1:18, FAA) for 24 hours. Samples were washed in steps with 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol I and 100% ethanol II respectively for 30 minutes each. Samples were dealcoholized in ethanol: xylol with a ratio 1:3, 1:1, 3:1, respectively, for 30 minutes. After that they were put in xylol: paraffin with ratio 1:9 for 24 hours at 57°C. The samples were infiltrated by replacing xylol/paraffin (1: 9) with pure paraffin for

embedding at 57°C for 24 hours. The block of samples were cut in longitudinal section by rotary microtome, following by staining with 1% safranin in 70% ethanol for 24 hours. The sections were observed under a light microscope for further analyses.

3. Results and discussion

3.1. Seed Germination and The Development of Protocorms

The germination frequency of *D. phalaenopsis* orchid seeds was observed five WASP on both NPCW and VWCW medium. About 37.45 % seeds were germinated on NPCW, this number was lower than that on VWCW medium which was about 58,03 % (Fig. 2). The nitrogen content in NP medium is very high (97.86%), compared to the nitrogen content in the VW medium (38.5%) and that it almost 3 times higher than in VW medium. Arditti (1967) stated that low concentrations of nitrogen stimulate germination of orchid seeds whereas high concentrations are inhibitory.

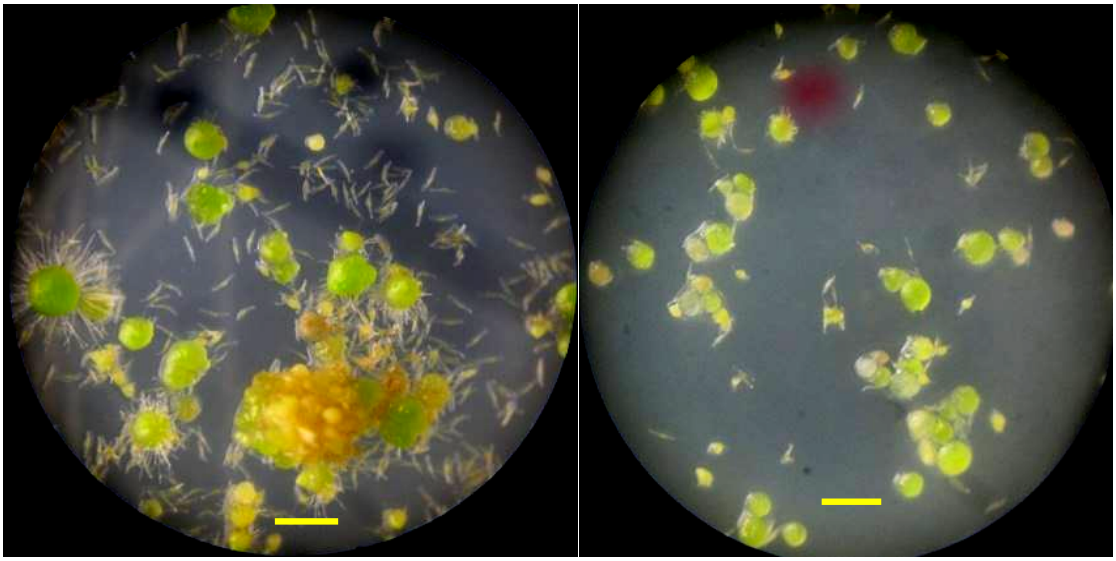

Figure 2. The growth of protocorm on NPCW (left) and VWCW (right) medium at 5 WASP (Bar : 1 mm). Germination percentage: NPCW 37.45%; VWCW 58.03%.

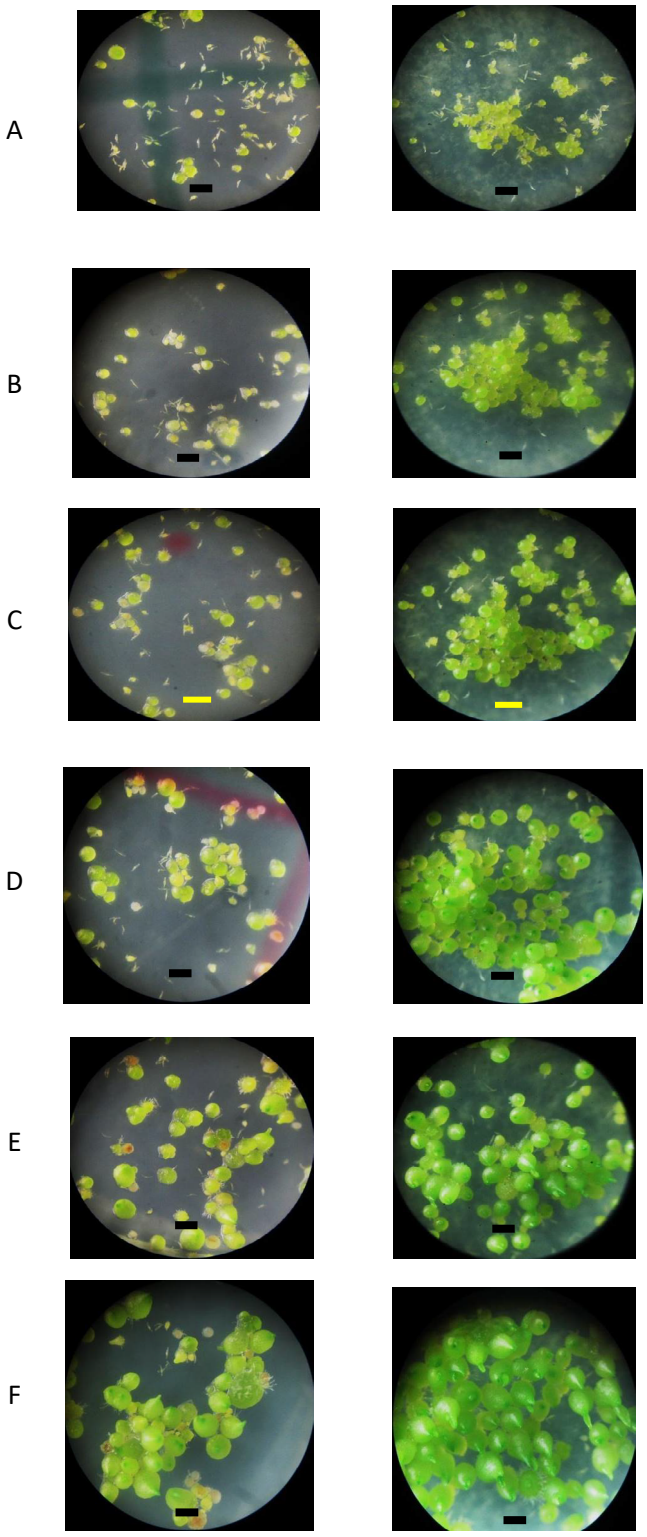

Figure 3. The growth of protocorms on VWCW (left) and VWCWTP (right) medium at 3 (A), 4 (B), 5 (C), 6 (D), 7 (E), and 8 (F) WASP (Bars: 1 mm).

It is consistance to the result of *in vitro* culture of orchid seed germination that was obviously affected by the type of basal medium (David *et al.,* 2015) and nitrogen source contained in the medium (Roy & Banerjee, 2002; Kauth *et al.* 2006; Stewart & Kane, 2006). Mineral reduction in medium will stimulate germination because of the high water requirement for germination (Rasmussen, 1995). The germination of *Vanilla planifolia* seed was greatly improved following a tenfold reduction in nitrogen content of Knudson's B medium, but was totally inhibited if concentrations were doubled (Arditti, 1967).

Germination of *Vanda helvola* in KC medium was higher than that in MS and VW medium. Nitrogen content in KC medium (16.04 mM) was lower than that in MS medium (60.01 mM) (Rasmussen, 1995 in David *et al.,*

2015) as well as *Geodorum densiflorum*, initiation of seed germination have no nutritional requirement (Roy & Banerjee, 2001). Protocorm and seedling of *D. phalaenopsis* orchid on VWCWTP medium was grown better than that on VWCW medium (Fig.3). The most prominent evidence was the color change of protocorm and seedling that turned the color from yellow into green and enlargement of the volume of protocorm (Table 1). Addition of organic substance such as tomato extract and peptone in culture medium (VWCWTP) could significantly increase the growth rate of embryo, protocorm and seedling on culture medium. Peptone as an amino acid stimulated the growth of protocorm on VWCWTP medium. The length and width of protocorms that were measured after 8 weeks cultured in VWCWTP medium were 160,67 ± 25,76 µm in length and 94,66 ± 8.3 µm in width and these were better than that cultured in VWCW medium which were 131,68 ± 31,66 µm in length and 81,91 ± 10,85 µm in width (Table 1). At 8-weeks after sowing, the seed germination frequency was also increased up to 94.42% in VWCWTP compared to that only 67.3 % on VWCW medium. It suggests that peptone and tomato extract on VW medium can promote the growth of protocorm. Peptone may play an important role on activation some genes related to the function of chlorophyll in the process of photosynthesis. However, tomato extract containing lycopene may act as antioxidant required as a trigger for initiation of embryo development, and this is in consistence to the results of increasing embryo development of *Phalaenopsis amabilis* orchids (Semiarti *et al.,* 2010).

Table 1. Germination percentage and size measurement of *D. phalaenopsis* orchid protocorm at 8 WASP at medium VWCW and VWCWTP

Medium	Σ Seed planted	Germination percentage at 8 WASP (%)	Protocorm measurement (µm) at 8 WASP	
			Length	Width
VWCW	1467	67.30	131.68 ± 31,66	81.91 ± 10.85
VWCWTP	1097	94.42	160.67 ± 25,76	94.66 ± 8.30

Organic additives such as tomato extract and peptone promote growth and development of seeds and regeneration of plantlets (Tawaro *et al.,* 2008). Tomato extract contains vitamin C, antioxidant and carotene that could affect the growth of embryo, especially during early stages of embryo development of orchids (Semiarti *et al.,* 2011). Tomato extract also contains K elements that facilitate the formation of water sac (micelles) within the cell walls, so it is easier to absorb water (George & Sherington, 1984). The easy way to absorb water will cause the embryo become more susceptible swelling (swollen) which was followed by the opening of testa and releasing embryos from the testa thereby accelerating the process of orchid seed germination (Dwiyani *et al.,* 2012). Peptone stimulates germination and growth in cultivation of seed orchid *Geodorum densiflorum* because it could be a major source of organic nitrogen and amino acid. Endogenous amino acid biosynthesis at early stage of protocorm development might not be adequate for healthy and faster growth of protocorm, therefore exogenous amino acid from peptone could enhance orchid seed germination and the growth of protocorm (Roy & Banerjee, 2001). Seed germination and *in vitro* seedling development of *Epidendrum ibaguense* Kunth. also need peptone as exogenous amino acid source (Hossain, 2008). Peptone has been reported for stimulating the growth of *Helichrysum bracteatum,* because amino acids can serve as a source of carbon and energy for the growth of plant cell. Furthermore, amino acid released the ammonia and organic acid. Organic acid then enter the Kreb's cycle, to be broken down to release energy through respiration. Amino acid is an available source of nitrogen, which can be taken by the cells more rapidly than in organic nitrogen form. Amino acid is also important for the biosynthesis of chlorophyll molecules which in turn affected carbohydrate content of the plant cell (Soad *et al.,* 2010).

3.2. Embryo Development Stages

The color of *D. phalaenopsis* orchid embryos in the first week after seed planting (WASP) was originally yellow, then gradually turned into green and swell (Fig. 4a and Fig.5b). Furthermore, during two weeks, embryos were grown into an ovoid-shape (Fig. 4b and Fig. 5c). During 2-3 weeks the seed coat was cracked and ovoid-shape embryos grew outside the seed coat (testa) and in the fourth week the embryo was spherical in shape and was separated from the seed coat. In the third week, the embryo formed protocorm (Fig. 4c and Fig. 5d, 5e). Shoot apical meristem (SAM) was formed at initial point of shoot development from protocorm at 4 WASP (Fig. 4d and 5f). Protocorm formed leaf primodium at 5 WASP (Fig. 4e and 5g) and first leaf at 8 WASP (Fig. 4g).

Orchid seeds germinated in an *in vitro* medium was preceded by embryos swell (Arditti, 1967) and changes color from yellow to green (Semiarti *et al.,* 2010). The step where the germinating embryo swells further and bursts out of the seed coat and an ovoid-shaped or spherical seedling was formed, was called the protocorm stage. On the upper flat surface of protocorm, a small protrusion was developed and developed as first leaf primordia. Absorbing hair was formed on the bottom of protocorm. Having formed the first leaves on protocorm was followed by the formation of roots (Arditti, 1967).

According to Semiarti *et al.* (2010), the developmental stage during the germination of *Phalaenopsis amabilis* orchid seed consist of six stages: the first stage is the embryo with yellow colour, the second stage is the embryo turns green in the first week or two, then the third stage is the embryo forms a bipolar structure (can be distinguished between the darker and lighter) at weeks three and four. At the fourth stage embryo began to form primordial leaves in the darker embryo at four weeks. At the fifth stage, protocorm already had two leaves at seven weeks and in the sixth stage protocorm had three leaves at eleventh up to twelfth weeks.

The germination of *D. phalaenopsis* orchid seeds at 5 WASP in VWCW medium was higher than in the NPCW medium (Fig. 2), it might because nitrogen content in the VW medium is lower (38.5%) than in the NP medium (97.86%). Nitrogen contents in the VW medium is only half level of the nitrogen content in the NP medium.

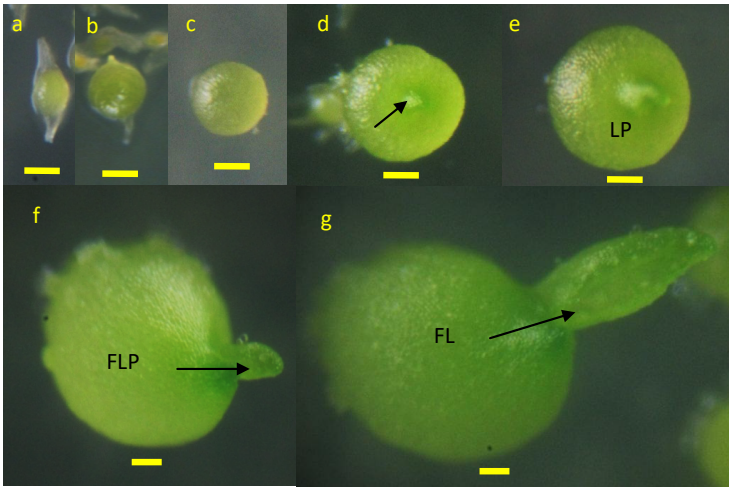

Figure 4. Embryo development stages of *Dendrobium phalaenopsis* orchid at VWCWTP medium. a. greening embryo (1 WASP), b. swollen embryo (2 WASP), c. protocorm (3 WASP), d. protocorm with shoot apical meristem (arrow) at 4 WASP, e. protocorm with leaf primordium (LP) (5 WASP), f. protocorm with first leaf primordium (FLP) at 6 WASP, g. protocorm with first leaf (FL) at 8 WASP

Dendrobium phalaenopsis orchid seed germination percentage was better in VWCWTP medium (94.42 % at 8 WASP) than that in VWCW medium (67.30% at 8 WASP) because VWCWTP medium is VW medium added with tomato extract and peptone. Organic additives like tomato extract and peptone promotes growth and development of seeds and regeneration of plantlets (Mondal *et al*., 2015). Tomato extract contains vitamin C, other antioxidant and carotene that could affect the growth of embryo, especially during the early stages of orchid embryo development (Semiarti *et al.,* 2011). Tomato extract (TE) also contains K elements that facilitate the formation of water sac (micelles) within the cell walls, so it is easier to absorb water (George & Sherington, 1984). The effect of peptone and tomato extract on the growth of embryo is related to the easy way of the embryo to absorb water as described by Dwiyani *et al.* (2012) in the development of *Vanda tricolor*. Roy and Banerjee (2001) reported that using peptone to stimulate germination on cultivation of *Geodorum densiflorum* orchid seeds obtained that peptone can be used as major source of organic nitrogen amino acid to induce cell development.

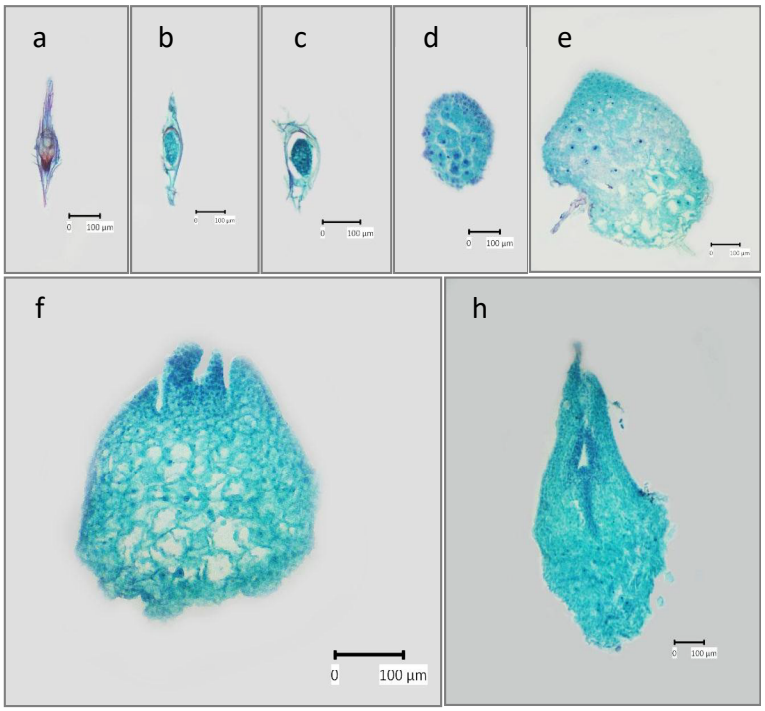

Figure 5. The developmental structure of Dendrobium phalaenopsis orchids embryo during embryo development. Anatomy of *D. phalaenopsis* orchids embryo. a. Phase 0, b. Phase 1, c. Phase 2, d. Phase 3, e. Phase 4, f. Phase 5, g. Phase 6 (Bars: 100 µm) .

4. Conclusion

Peptone as amino acid source and tomato extract as antioxidant plus sugar as energy source in macronutrient effectively induced seed germination and accelerated the growth rate of early stage of embryo development of *D. phalaenopsis*, increased the number of seed germination and the growth of protocorms onVW basic medium.

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