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The Effects of Light and a Combination of Growth Regulators on the Induction of Somatic Embryogenesis in Orchid *Rhynchostylis gigantea* (LindI.) Ridl.

(Kesan Cahaya dan Gabungan Pengawalatur Pertumbuhan terhadap Induksi Embriogenesis Somatik pada Orkid *Rhynchostylis gigantea* (LindI.) Ridl.)

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ABSTRACT

The squirrel-tail orchid (*Rhynchostylis gigantea*) belongs to the Orchidaceae family. This orchid is indigenous to Southeast Asia and is scented and arranged in a bouquet of dangling stems. Its uniqueness makes it commercially desirable. *In vitro* culture has been utilized for orchid proliferation for a very long time to aid in the development of orchid seedlings. However, not all orchid species react the same way. Through somatic embryogenesis without gamete fusion, *in vitro* culture techniques can produce new plants during the entire embryonic phase. Endogenous and exogenous hormones, as well as light, influence the success of embryogenesis. Determining the optimal environmental parameters (light, combination and concentration of growth regulators, and their interaction) for inducing somatic embryogenesis from leaf explants in *R. gigantea* is the purpose of the present study. The leaves of *R. gigantea* clone 19 were utilized as explants in this investigation, utilizing a completely random factorial design. The first factor is a combination of growth regulator types and concentrations, and the second factor is light. Explants that are alive and growing at a rapid rate indicate that the in vitro culture is successful. Light and the combination of growth regulators significantly affected the percentage of viable explants, the beginning date of callus formation, and the number of embryogenic calluses produced. The interaction of two components (light and a combination of growth regulators) did not affect the three characteristics, with the exception of the proportion of somatic embryogenesis. Incubation in the dark is the optimal environment for initiating somatic embryogenesis in explants. The optimal combination of plant growth regulators for inducing somatic embryogenesis was 0.5 mgL^{-1} TDZ and 0.1 mgL^{-1} BAP. Bright light and a concentration of 1.0 mgL^{-1} TDZ + 0.1 mgL^{-1} BAP were the optimal interaction conditions for the induction of somatic embryogenesis in *R. gigantea*.

Keywords: Dark; light; *Rhynchostylis gigantea;* somatic embryo

ABSTRAK

Orkid *Rhynchostylis gigantea* ialah ahli famili Orchidaceae Orkid ini berasal dari Asia Tenggara, wangi dan tersusun dalam sejambak batang berjuntai. Keunikan menjadikan item itu diingini secara komersial. Kultur *in vitro* telah digunakan untuk pembiakan orkid untuk masa yang sangat lama untuk membantu dalam pembangunan anak benih orkid. Walau bagaimanapun, tidak semua spesies orkid bertindak balas dengan cara yang sama. Melalui embriogenesis somatik tanpa gabungan gamet, teknik kultur *in vitro* boleh menghasilkan tumbuhan baharu semasa keseluruhan fasa embrio. Hormon endogen dan eksogen, serta cahaya, mempengaruhi kejayaan embriogenesis. Menentukan parameter persekitaran optimum (cahaya, gabungan dan kepekatan pengawalatur pertumbuhan dan interaksinya) untuk mendorong embriogenesis somatik daripada eksplan daun dalam *R. gigantea* adalah tujuan kajian ini. Daun klon *R. gigantea* 19 telah digunakan sebagai eksplan dalam kajian ini, menggunakan reka bentuk faktorial rawak

sepenuhnya. Faktor pertama ialah gabungan jenis dan kepekatan pengawalatur pertumbuhan dan faktor kedua ialah cahaya. Eksplan yang hidup dan berkembang pada kadar yang cepat menunjukkan bahawa kultur *in vitro* berjaya. Faktor cahaya dan gabungan pengawalatur pertumbuhan memberi kesan ketara kepada peratusan eksplan yang berdaya maju, masa permulaan kalus dan bahagian kalus embriogenik yang dihasilkan. Interaksi dua komponen (cahaya dan gabungan pengawalatur pertumbuhan) tidak mempunyai kesan ke atas ketiga-tiga ciri ini, dengan pengecualian bahagian embriogenesis somatik. Pengeraman dalam gelap adalah persekitaran yang optimum untuk memulakan embriogenesis somatik dalam eksplan. Gabungan optimum pengawalatur pertumbuhan tumbuhan untuk mendorong embriogenesis somatik ialah 0.5 mgL^{-1} TDZ dan 0.1 mgL^{-1} BAP. Cahaya terang dan kepekatan 1.0 mgL^{-1} TDZ + 0.1 mgL^{-1} BAP adalah keadaan interaksi optimum untuk induksi embriogenesis somatik dalam *R. gigantea*.

Kata kunci: Embrio somatik; gelap; *Rhynchostylis gigantea*; terang

Introduction

Orchids are one of the most popular ornamental plants beloved by many people. Over 10% of the flowering plant species, or approximately 30,000 species, belong to this family of decorative plants (Almeida et al. 2017). Indonesia has at least 5,000 species of orchids (Comber 1990), but only a few of these species are cultivated and popular. The *Phalaenopsis* sp., *Cymbidium* sp., *Vanda* sp., *Dendrobium* sp., and *Rhynchostylis* sp. are the few that have been identified and nurtured. Meanwhile, majority of the others have yet to be discovered and cultivated while possessing great promise.

Rhynchostylis (Lindl) Ridl is an orchid genus indigenous to Southeast Asia (Vietnam, Laos, Kalimantan, Malaysia, and Thailand). The woodlands of Thailand are home to three species of this genus: *Rhynchostylis gigantea*, *Rhynchostylis retusa*, and *Rhynchostylis coelestis* (Prasongsom, Thammasiri & Chuenboonngarm 2014). *R. gigantea*, known as the foxtail orchid, is a species of orchid indigenous to Thailand. In its natural habitat, i.e., the tropical rainforest and mixed forest, *R. gigantea* is epiphytic. These orchids bloom in midwinter (December to February) (Kaewkhiew & Kaewduangta 2010) and feature a bouquet shaped like a squirrel's tail, consisting of pendulous stalks that flower freely from the base of the stems. This distinguishes *R. gigantea* from all other orchid varieties (Obsuwan, Yoodee & Uthairatanakij 2010). It has a distinctive blossoming with a frayed stalk from the main stem, where the bucket is comprised of more than 50 flowers, with a powerful fragrance once it blooms (Neera & Boungphengphanh 2016). This orchid's distinctiveness is its greatest asset; hence, its commercialization is highly viable.

R. gigantea is not extensively cultivated in Indonesia due to propagation difficulty. One of the reasons orchids are difficult or unusual to germinate in their native habitat is that their seeds lack endosperm and require the assistance of mycorrhiza to convert starch into sugar (Ramasoot, 2017). Additionally, the extremely limited variety of *R. gigantea* demands expansion. Hence, viable alternatives, such as *in vitro* culture with somatic embryogenesis, are required for plant propagation and the expansion of *R. gigantea*.

In vitro culture is one method for rapid multiplication and producing uniform progeny from orchid plants. Somatic embryogenesis is one of the *in vitro* cultivation techniques by which somatic cells create new plants during the embryonic phase without gamete fusion (Rose & Song 2017). Compared to other *in vitro* culture techniques, the somatic embryogenesis approach for propagating orchids produces more seeds. It is one of the first steps in increasing plant diversity through mutation and directional selection (Rachmawati et al. 2020). *Phalaenopsis* sp. (Rianawati et al. 2009; Van Mint 2018), *Epipactis veratrifolia* (Moradi et al. 2017), *Vanda tesselata* Roxb. Hooks. (Manokari, Latha & Shekhawat 2021), and *Dendrobium* 'Balithi CF22-58' have been previously propagated utilizing somatic embryogenesis procedures (Rachmawati et al. 2020). While somatic embryogenesis has been performed on *Rhynchostylis*, there have been only two reports: *R. gigantea* with mutant white flowers (Li & Xu 2009) and *R. gigantea* native to Vietnam with a blood-red hue (Van Minh 2019).

In vitro culture has been utilized for decades in the multiplication of orchids, although not all orchid species exhibit the same reaction to given conditions (Ramasoot 2017). According to Neera and Boungphengphanh (2016), several factors, including medium nutrients, growth regulators, explants, and other environmental factors, can affect the induction and regeneration of plants grown via *in vitro* culture. Cytokinin is one of

the several growth regulators that play a crucial role in embryogenesis. The use of cytokinins alone or in combination with other growth regulators, such as thidiazuron (TDZ) and benzyl amino purine (BAP), has been applied to many plant species (Hong, Chen & Chang 2010). The formation of somatic embryos in *Phalaenopsis amabilis* L. was optimal with the addition of 1 mgL^{-1} TDZ alone (Mose et al. 2017), while in the *Dendrobium* 'Balithi CF22-58' orchid group, 1.5 mgL^{-1} TDZ + 0.5 mgL^{-1} BAP produced the best embryogenic callus formation (Chen & Chang 2004).

The light treatment administered has a substantial impact on the performance of the growth regulators in the explants. Furthermore, the interaction between the light and growth regulators can influence the growth and development of the explants. Generally, the creation of callus and somatic embryos occurs in dark settings, as shown by the findings of Sedaghati, Haddad and Bandehpour (2018) on *Portulaca oleracea* L., which produced more somatic embryos with a 3-week dark treatment compared to a photoperiod of 16 h light/8 h dark. The optimal propagation of *R. gigantea* through somatic embryogenesis necessitates a suitable treatment based on the results obtained from various plant species and treatments. This study aimed to identify the optimal interaction between the types and concentrations of growth regulators and light. In addition, the optimal environmental parameters (light, combination and concentration of growth regulators, and their interactions) for triggering somatic embryogenesis from leaf explants in *R. gigantea* were also determined.

Materials and Methods

Plant materials and experimental design

This study used a completely randomized factorial design (CRD), and the leaves of *R. gigantea* clone 19 as explants. The first factor was a mixture of growth regulator types and doses, specifically 0, 0.1, 0.2, and 0.5 mgL^{-1} of BAP and 0, 0.5, 0.75, and 1.0 mgL^{-1} of TDZ. Meanwhile, the second factor was lighting, specifically light and dark conditions. A total of 32 treatments were repeated 3 times, with 3 units for each treatment, making up to 288 units. The growth medium contained half-strength MS (½ MS). The abaxial portion of the leaf explants measuring 1-1.5 cm was sliced. The leaves were placed in culture bottles with the abaxial part of the media attached. Each bottle contained three young explants, and the culture bottles were incubated at 20 °C. The light-treated explants were incubated for 24 h in a chamber with a 1200 lux LED light, while the dark-treated explants were placed in a dark box.

Observable variables

Explant growth was monitored every two weeks based on the following characteristics: 1) percentage of live explants (%), determined by dividing the number of live explants by the total number of those cultured. According to Parthibhan et al. (2018), dead explant indicators include browning and blackening of the explant surface. 2) Callus initiation time, days after planting (DAP), i.e., the number of days from explant planting to callus development. 3) The percentage (%) of callus created and the number of callus, prospective shoots, and shoots (%), where observations of the development of callus, prospective shoots, and shoots were conducted two weeks after planting (WAP). Meanwhile, the number of calluses, prospective shoots, and shoots was observed at 14 WAP. 4) Observation of somatic embryo morphology with a camera. The observed morphology included color, texture of the callus, and the stages of somatic embryogenesis (globular, heart, and torpedo). Calluses with successfully formed shoots were transferred to the regeneration medium, which consisted of ½ MS plus 1 mgL^{-1} BAP and 0.5 mgL^{-1} NAA.

Data analysis

Observational data were analyzed descriptively with SPSS version 25 (IBM SPSS Statistics 25, 2020), with the 5% F test for analysis of variance (ANOVA). More tests on considerably distinct characters were conducted using Duncan's Multiple Range Test (DMRT).

Results and Discussion

The large percentage of living explants and their development demonstrate the success of *in vitro* cultivation. Additionally, the influence of light intensity and the combination of growth regulators appear to significantly impact the percentage of viable explants, the duration required for callus formation, and the proportion of embryogenic cells in *R. gigantea*. The interplay of the two elements (light and mixture of growth regulators) did not affect these three characteristics, except the rate of somatic embryogenesis (ANOVA not shown).

PERCENTAGE OF LIVING EXPLANTS

Observation of the percentage of living explants was more significant in the dark than in the light (Figure 1(a)). Light is one of the most influential environmental elements in the number of explants surviving. Different light conditions provided to explants might influence the availability of endogenous hormones, resulting in the browning of explants. This causes the number of live explants to continue decreasing each week. Bright light resulted in a lower percentage of viable explants than dark light at 14 WAP.

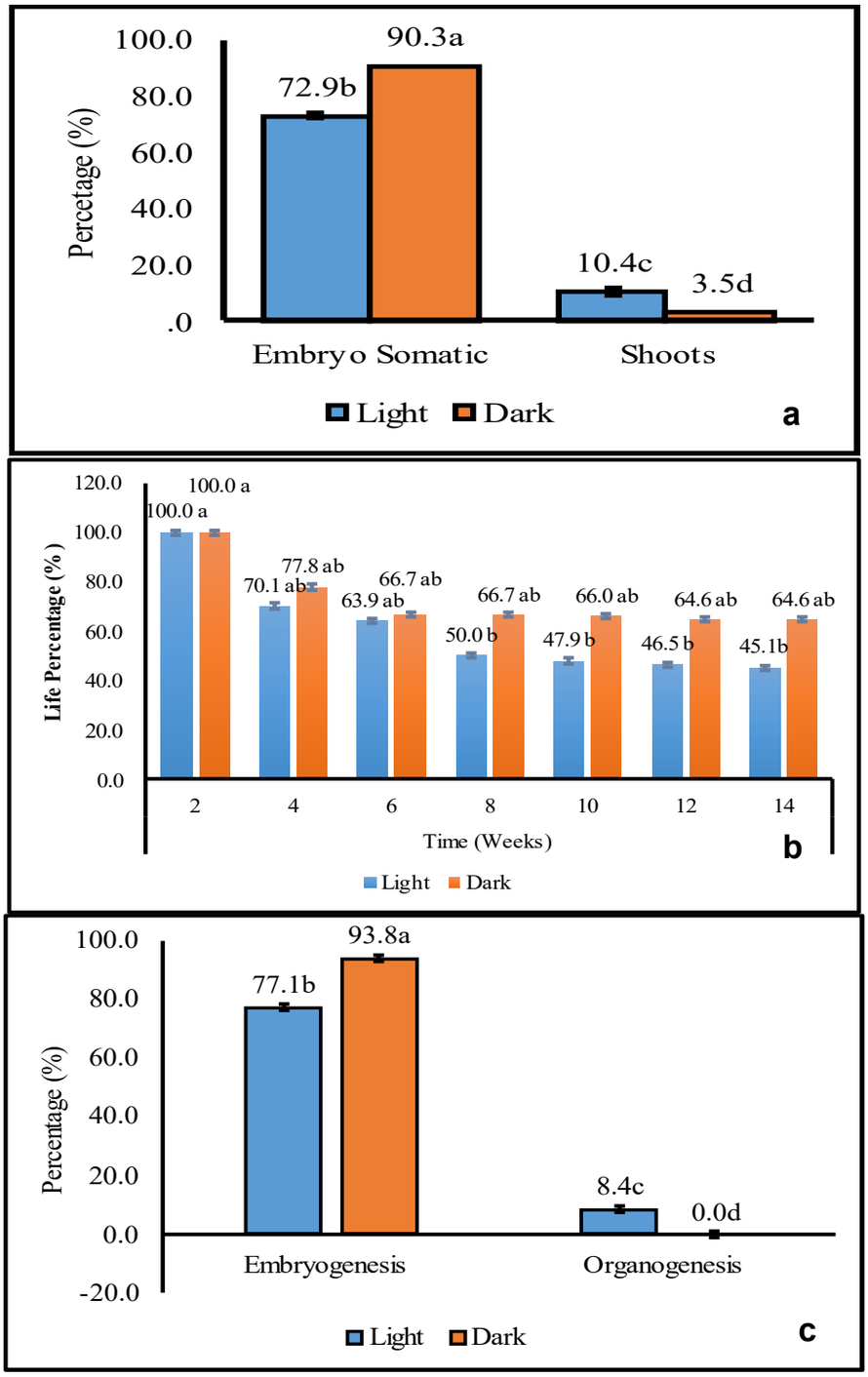


FIGURE 1. (a) Graph of the mean percentage of live explants; (b) percentage of explants forming somatic embryos and shoots; (c) percentage of development through embryogenesis and organogenesis after 14 WAP

The availability of endogenous explant hormones, including auxin, is affected by environmental conditions with high light intensity (brightness). Endogenous auxin is highly light sensitive; this hormone will rise with low or dark light intensity (Chen et al. 2019). Presumably, the light-treated explants contained less endogenous auxin, which could impede the growth and development of explants, causing them to perish quickly. In addition to influencing the presence of endogenous hormones in explants, light conditions influence the browning of explants. Generally, browning is caused by the enzyme polyphenol oxidase, which contains phenolic chemicals. The levels of the resultant phenolic compounds, such as flavanols, will increase once affected by light (Chen et al. 2019).

Furthermore, exogenous hormones had an impact on the proportion of live explants. Table 1 shows that a significant proportion of viable explants (90.7%) was observed in the combination of growth regulators 1 mgL^{-1} TDZ and 0.2 mgL^{-1} BAP (combination 15), with TDZ concentrations higher than BAP. Compared to BAP, TDZ plays a much more significant role in the growth and development of explants. Even at high TDZ concentrations (1 mgL^{-1}), *R. gigantea* leaf explants grew normally. However, this is in contrast to the study by Hoesen et al. (2008) on *Dendrobium lineale*, in which 0.5 mgL^{-1} TDZ resulted in the highest percentage of live explants (57.27%) and 1.0 mgL^{-1} TDZ resulted in the lowest average value (41.07%). This behavior is possible because TDZ is a potent form of cytokinin, and supplementation of TDZ in high quantities inhibits explant development. At this juncture, each plant will respond differently to the offered growth regulators.

Although the combinations of 1 mgL^{-1} TDZ and 0.2 mgL^{-1} BAP resulted in the highest percentage of surviving explants, the results did not differ significantly compared to several other treatments that used 0.5 mgL^{-1} TDZ and 0.1-0.5 mgL^{-1} BAP (combinations 6, 7, 8, and 10). The lowest proportion is seen in the control (combination 1), which lacked both growth regulators. In addition to the media, it is hypothesized that additional factors influence the viability of live explants, such as the selection of explants employed. Young or meristematic explants have a strong capacity for regeneration, allowing them to sustain growth and development (Chen & Chang 2006). Also, the presence of endogenous and exogenous hormones in the media will affect the growth and development of explants lacking appropriate growth regulators, resulting in a poor or nonexistent response to growth and development (Yanti & Isda 2021).

TABLE 1. The average of live explants (%) and callus initiation time (day after planting/DAP) for leaf explants of *R. gigantea* treated with light and growth regulators

	Treatment	Live explants (%)	Callus initiation (DAP)
Lighting			
1	Bright	45.1±4.3^{a}	50.6±2.41^{a}
2	Dark	64.6±4.5^{b}	39.2±2.56^{b}
Combination of growth regulators (mgL^{-1})			
1	0 TDZ + 0 BAP	29.4±1.0^{c}	0±0.0^{a}
2	0 TDZ + 0.1 BAP	48.3±2.4^{c}	0±0.0^{a}
3	0 TDZ + 0.2 BAP	62.5±3.7^{bc}	43.8±8.1^{c}
4	0 TDZ + 0.5 BAP	68.3±11.1^{bc}	47.3±8.8^{bc}
5	0.5 TDZ + 0 BAP	69.1±5.0^{bc}	39.2±3.2^{c}
6	0.5 TDZ + 0.1 BAP	85.1±8.8^{a}	33.0±0.6^{c}
7	0.5 TDZ + 0.2 BAP	80.0±4.0^{a}	39.3±3.2^{c}
8	0.5 TDZ + 0.5 BAP	83.2±2.4^{a}	39.0±3.1^{c}
9	0.75 TDZ + 0 BAP	59.8±4.3^{bc}	41.2±1.9^{c}
10	0.75 TDZ + 0.1 BAP	81.9±4.6^{a}	43.0±0.5^{c}
11	0.75 TDZ + 0.2 BAP	68.2±6.9^{bc}	43,5±1.3^{c}
12	0.75 TDZ + 0.5 BAP	77.7±5.6^{abc}	43.0±1.0^{c}
13	1.0 TDZ + 0 BAP	54.7±3.3^{bc}	40.5±2.5^{c}
14	1.0 TDZ + 0,1 BAP	74.7±1.2^{abc}	37.8±0.7^{c}
15	1.0 TDZ + 0.2 BAP	90.7±4.6^{a}	36.8±1.2^{c}
16	1.0 TDZ + 0.5 BAP	69.8±15.3^{bc}	39.5±1,7^{c}

The 5% DMRT test showed a significant difference between the average value followed by different letters in the same column

TIME FOR CALLUS INITIATION

Indirect somatic embryogenesis begins with the formation of a callus and progresses to the next stage. Table 1 shows that the dark condition also produced a shorter callus initiation time than light exposure, while combination 6 generated the quickest callus initiation time. Callus formation typically occurs in low-light or dark environments. This is because light can affect endogenous auxin pathways in explants (Liang et al. 2020), and dark incubation conditions can produce more endogenous auxin (Sedaghati, Haddad & Bandehpour 2018). Meanwhile, the presence of the right endogenous and exogenous hormones (TDZ and BAP) can speed up the formation of callus explants in the dark (Novak, Luna & Gamage 2014).

The combination of growth regulators significantly affects callus formation, callus initiation, and callus volume. The combination of growth regulators 1 mgL^{-1} TDZ and 0.2 mgL^{-1} BAP resulted in the shortest average time to callus formation (33 DAP, Table 1). This is consistent with the findings of Popilia, Linalatil and Devi Rohmah (2021), who reported that the addition of 0.5 ppm TDZ accelerated the time required for callus formation in pomegranate seed explants. In this experiment, although the combination of 0.5 ppm TDZ and 0.1 ppm BAP in combination 6 was the lowest concentration among the other combinations, it was the most effective at accelerating callus formation from *R. gigantea* leaf explants. Lower TDZ concentrations promote callus formation more quickly than higher concentrations. Since TDZ is a potent cytokinin, excessive amounts can inhibit the growth and development of explants.

In contrast, the study by Mose et al. (2017) on *Phalaenopsis amabilis* with a TDZ concentration of 1–3 ppm alone resulted in the best somatic embryo formation. Depending on the species, type of plant, medium, concentration of endogenous hormones, and other environmental factors, the combination of growth regulators can produce different responses (Guo & Jeong 2021). Media without growth regulators or using 0.1 mgL^{-1} BAP could not promote the growth of all explants, as observed in Table 1. Presumably, without the addition or slight addition of exogenous growth regulators and low endogenous hormone content, the formation of callus was impossible. The presence of adequate endogenous and exogenous hormones for cell division and differentiation can aid in the growth of callus (Vondrakova et al. 2018).

PERCENTAGE OF SOMATIC EMBRYOGENESIS AND ORGANOGENESIS

Figure 1(b) shows that dark treatment produced larger somatic embryos (90.3%) than light (72.9%), and this condition is in line with the results of *Epicpatis veratrifolia* (Moradi et al. 2017). The formation of more somatic embryos in dark conditions will positively impact explants in terms of protein accumulation and reduced production of phenolic compounds (Moradi et al. 2017). Protein production in explants can support the provision of nutrients for explant development and the formation of somatic embryos. Meanwhile, the production of phenolic compounds can occur in light or dark conditions, but in general, light conditions affect the formation of phenolic compounds more (Jang, Ho & Park 2020). Explants with higher levels of phenolic compounds are more likely to become brown and necrotize, which prevents the development of calluses and somatic embryos and results in explant death (von Aderkas et al. 2015).

R. gigantea leaf explants underwent a process of organogenesis by producing adventitious shoots. Organogenesis is the formation of plant organs in the form of roots or shoots from the explants. The process of organogenesis only occurs in bright conditions (Figure 1(c)). Leaf explants of *Scaevola sericea* experienced the formation of adventitious shoots in sunny conditions (Liang et al. 2020). The percentage of shoots formed was also more significant in bright conditions than in dark conditions because light could assist the process of explant morphogenesis, i.e., in the form of shoots, roots, or leaves on explants (Khotskova et al. 2018).

Endogenous growth regulators influence the establishment of somatic embryogenesis (ANOVA not shown). Table 2 shows that the highest formation of somatic embryos was obtained from the addition of 0.5 mgL^{-1} TDZ alone or in combination with BAP at lower concentrations. Growth regulators can replace the auxin/auxin-cytokinin interactions needed during the formation of somatic embryos because TDZ can modulate the formation of endogenous hormones in the form of auxin or other cytokinins (Chhabra et al. 2008). The formation of high somatic embryos in *Primulina tobacum* is influenced by higher concentrations of TDZ than BAP (Ma et al. 2010). Furthermore, the addition of TDZ to *Phalaenopsis amibilis* var. Formosa is effective for direct induction of embryogenesis (Chen & Chang 2004). Optimum somatic embryo production was observed in *P. amibilis* using TDZ (Moradi et al. 2017; Mose et al. 2020).

Organogenesis occurred in explants treated with a single BAP but with a lower percentage than the formation of somatic embryogenesis. This may occur due to higher concentrations of BAP, which can lead to the formation of adventitious shoots (Ma et al. 2010). However, in some cases, somatic embryogenesis can also occur with the addition of a higher BAP concentration (Moradi et al. 2017).

The interaction of light treatment and a combination of growth regulators significantly affected the characteristic of somatic embryo formation in *R. gigantea* leaf explants (ANOVA not shown). A higher number of somatic embryos were produced in almost all combinations of growth regulators in the dark condition. In other words, the somatic embryo grew well on media containing TDZ growth regulators with a concentration of 0.5–1.0 mgL^{-1} and 0.1–0.5 mgL^{-1} BAP, whereas in bright light, somatic embryos were produced well in the combination of 0.5 mgL^{-1} TDZ and 0.1–0.5 mgL^{-1} BAP (combinations 5–8), 0.75 mgL^{-1} TDZ and 0.1 mgL^{-1} BAP (combination 10), and 0.75 mgL^{-1} TDZ plus 0.5 mgL^{-1} BAP or 1 mgL^{-1} TDZ plus 0.1–0.5 mgL^{-1} BAP (combinations 12–16, Table 2). The interaction treatment that did not produce embryogenic callus was the combination of growth regulators 1 (without TDZ and BAP). In this interaction, the absence of exogenous hormones is thought to affect somatic embryo formation significantly. However, endogenous explant hormones are insufficient to trigger somatic embryo formation, especially with application to bright conditions, which generally affect the formation of phenols, inhibiting somatic embryo formation.

TABLE 2. The effects of a combination of growth regulators on the percentage of somatic embryogenesis and organogenesis, as well as the interaction of light and growth regulators in *R. gigantea* leaf explants during the formation of somatic embryogenesis at 14 WAP (week after planting)

No.	Combination of growth regulators	Explant development		Interaction of lighting and growth regulators on somatic embryo formation	
		Somatic embryogenesis	Organogenesis	Light	Dark
1	0 TDZ + 0 BAP	16.5±7.4^{c}	0.0±0.0^{b}	0.0±.0.0^{d}	33.0±0.0^{c}
2	0 TDZ + 0.1 BAP	50.0±7.6^{d}	33.5±0.0^{a}	33.0±0.0^{c}	67.0±0.0^{b}
3	0 TDZ + 0.2 BAP	83.5±7.4^{b}	0.0±0.0^{b}	67.0±0.0^{b}	100.0±0.0^{a}
4	0 TDZ + 0.5 BAP	66.5±14.9^{c}	33.5±0.0^{a}	33.0±0.0^{c}	100.0±0.0^{a}
5	0.5 TDZ + 0 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
6	0.5 TDZ + 0.1 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
7	0.5 TDZ + 0.2 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
8	0.5 TDZ + 0.5 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
9	0.75 TDZ + 0 BAP	66.5±14.9^{c}	0.0±0.0^{b}	33.0±0.0^{c}	100.0±0.0^{a}
10	0.75 TDZ + 0.1 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
11	0.75 TDZ + 0.2 BAP	83.5±7.4^{b}	0.0±0.0^{b}	67.0±0.0^{b}	100.0±0.0^{a}
12	0.75 TDZ + 0.5 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
13	1.0 TDZ + 0 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
14	1.0 TDZ + 0,1 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
15	1.0 TDZ + 0.2 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}
16	1.0 TDZ + 0.5 BAP	100.0±0.0^{a}	0.0±0.0^{b}	100.0±0.0^{a}	100.0±0.0^{a}

The 5% DMRT test showed a significant difference between the average value followed by different letters in the same column

MORPHOLOGY OF SOMATIC EMBRYOS

Color is one of the most distinct morphological differences observed between the somatic embryos produced under dark and light treatments. Figure 2 (1-4) shows that the calluses produced from explants incubated in bright light are green, while those incubated in the dark are yellowish-white (Figure 2 (5-8)). The presence of light causes the formation of chlorophyll grains in the callus, resulting in a greener appearance than in the absence of light.

Figure 2 shows that the stages of somatic embryos formed in light or dark conditions looked similar. They were in the pro-embryo, globular, scutella, and coleoptile stages (Yan et al. 2020). The callus appeared solid and round in the pro-embryo phase, with small spheres, thick walls, and large nuclei (Mose et al. 2017). The pro-embryo will then continue to develop and enlarge to form a globular embryo, distinguished by the formation of dense, yellowish-green, shiny, and thin-walled round nodules (Van Mint 2018). The globular embryo will continue to develop into a scutellum and then elongate into a coleoptile. During the coleoptile phase, the root and stem meristems can differentiate, and the vascular system begins to grow (Yeung 2017).

In addition to light, growth regulators influence the differences between the somatic embryos formed. Observations at 14 WAP revealed the formation of three stages of somatic embryos in the explants: callus, bud candidates, and shoots, as seen in Table 3. The combination of growth regulators 7 and 6 under light and dark conditions produced the highest average number of callus formations. Figures 3(a) (7–10) and 3(b) (2, 6, and 8) show that the calluses formed on the explants are embryogenic, which develop into subsequent stages of somatic embryogenesis. Furthermore, the embryogenic calluses that further developed into other phases of somatic embryos had a yellowish-green hue and a crumbly or dry-to-compact consistency (Van Mint 2018.

Upon observation at 14 WAP, prospective shoots represent the final stages of somatic embryogenesis. After the formation of the embryogenic callus, the callus underwent globular, scutellar, and coleoptilar stages of development before starting the shoot-forming tissue. Table 3 shows that the combination of different growth regulators produced the most significant average number of prospective shoots under light and dark conditions. At this stage, embryos were fully developed, and root, stem, and vascular meristems could be distinguished (Yeung 2017). The terminal shoot candidates have a meristematic morphology that will continue to divide and form plantlet-like structures, as seen in Figure 3(a) (12–15) and 3(b) (3–4 and 13–15).

Under light conditions, the combination of growth regulators 2 (0.1 ppm BAP) and 4 (0.5 ppm BAP) resulted in the formation of shoots (Table 3). In bright conditions, the combination of growth regulators 2 and 4 that contain BAP resulted in the formation of shoots. At certain concentrations, self-applied BAP can induce shoot formation (Sushmarani, Venkatesha & Deekska 2021). On the other hand, in the dark condition, combination 14, which contained BAP and TDZ, affected the shoot formation. This is possible because TDZ is a cytokinin that plays a role in cell division and induces budding. Depending on the species, type, and age of explants, concentrations of endogenous hormones, and environmental treatment, combinations of growth

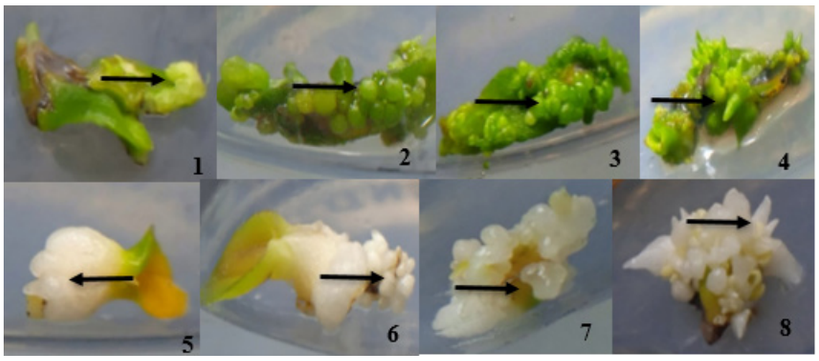


FIGURE 2. The stages of somatic embryo formation and development in of *R. gigantea* leaf explants at 14 WAP in light (1-4) and dark (5-8) conditions. Pro-embryo phase (1 & 5), globular (2 & 6), scutellar (3 & 7), and coleoptile (4 & 8)

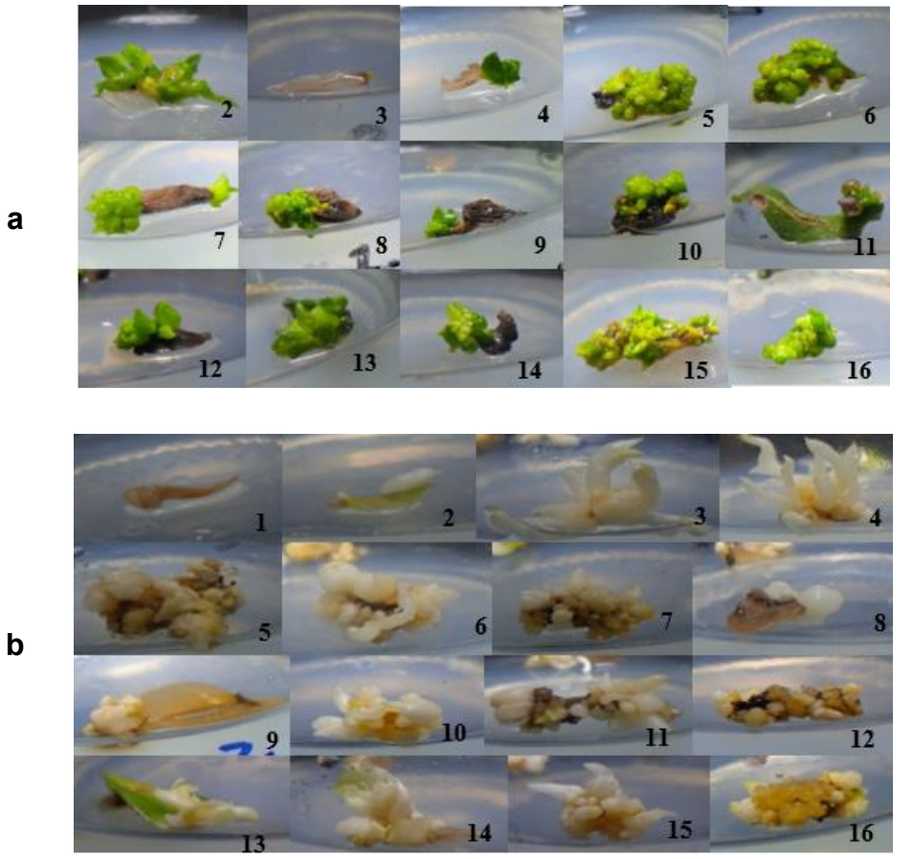
regulators and other therapies can elicit different responses, as indicated by differences in explant morphology resulting from growth and development.

Figure 4(a) shows the development of plantlets resulting from somatic embryogenesis (and grown on a regeneration medium) after 6 months, Figure 4(b) shows a regenerated young *R. gigantea* plant after 20 months, and Figure 4(c) shows *R. gigantea* plants producing flowers after 60 months.

TABLE 3. Mean number of calluses, shoots candidates, and shoots on *R. gigantea* leaf explants at 14 WAP

Combination of growth regulators	Light			Dark		
	Callus	Candidate shoots	Shoots	Callus	Candidate shoots	Shoots
0 TDZ + 0 BAP	0.0±00^{d}	0.0±0.0^{d}	0.0±0.0^{c}	0.3±0.3^{c}	0.0±0.0^{c}	0.0±0.0^{c}
0 TDZ + 0.1 BAP	0.7±0.7^{cd}	0.0±0.0^{d}	3.0±2.6^{a}	1.0±0.6^{bc}	0.0±0.0^{c}	0.0±0.0^{c}
0 TDZ + 0.2 BAP	1.3±0.8^{c}	0.0±0.0^{d}	0.0±0.0^{c}	0.0±0.0^{d}	9.7±1.3^{a}	0.0±0.0^{c}
0 TDZ + 0.5 BAP	0.3±0.3^{cd}	0.0±0.0^{d}	1.3±1.1^{b}	1.0±0.6^{bc}	7.3±2.3^{ab}	0.0±0.0^{c}
0.5 TDZ + 0 BAP	0.7±0.3^{cd}	1.7±2.0^{bc}	0.0±0.0^{c}	5.3±1.8^{ab}	0.7±0.7^{bc}.	0.0±0.0^{c}
0.5 TDZ + 0.1 BAP	2.0±1.2^{b}	2.0±3.4^{b}	0.0±0.0^{c}	6.3±0.7^{a}	0.0±0.0^{c}	0.0±0.0^{c}
0.5 TDZ + 0.2 BAP	6.3±2.6^{a}	0.3±0.6^{d}	0.0±0.0^{c}	4.7±2.0^{b}	0.0±0.0^{c}	0.0±0.0^{c}
0.5 TDZ + 0.5 BAP	4.0±0.6^{a}	1.0±1.6^{c}	0.3±0.6^{c}	6.0±2.0^{a}	0.0±0.0^{c}	0.0±0.0^{c}
0.75 TDZ + 0 BAP	0.3±0.3^{cd}	0.0±0.0^{d}	0.0±0.0^{c}	2.6±0.9^{b}	3.3±2.8^{b}	0.0±0.0^{c}
0.75 TDZ + 0.1 BAP	2.0±0.0^{b}	0.7±1.1^{c}	0.0±0.0^{c}	4.0±2.3^{ab}	3.0±3.0^{b}	0.3±0.3^{b}
0.75 TDZ + 0.2 BAP	1.0±0.6^{c}	0.0±0.0^{d}	0.0±0.0^{c}	3.3±0.7^{b}	0.0±0.0^{c}	0.0±0.0^{c}
0.75 TDZ + 0.5 BAP	2.7±1.8^{b}	1.0±1.7^{b}	0.0±0.0^{c}	2.7±0.9^{b}	1.0±0.6^{bc}	0.0±0.0^{c}
1.0 TDZ + 0 BAP	1.7±0.3^{bc}	2.3±2.0^{b}	0.0±0.0^{c}	1.3±0.8^{bc}	1.0±1.0^{bc}	0.0±0.0^{c}
1.0 TDZ + 0,1 BAP	0.7±0.3^{cd}	1.0±1.0^{c}	0.3±0.6^{c}	1.7±1.2^{bc}	2.0±1.1^{bc}	1.0±0.6^{a}
1.0 TDZ + 0.2 BAP	0.3±0.3^{cd}	4.3±5.1^{a}	0.3±0.6^{c}	4.0±1.5^{ab}	2.7±1.4^{b}	0.0±0.0^{c}
1.0 TDZ + 0.5 BAP	2.0±1.6^{b}	1.0±1.0^{c}	0.0±0.0^{c}	1.7±0.9^{bc}	2.3±1.8^{bc}	0.0±0.0^{c}

The 5% DMRT test showed a significant difference between the average value followed by different letters in the same column



The numbers in the image represent the treatment (combination) codes of the growth regulators

FIGURE 3. The morphology of calluses grown in of *R. gigantea* leaf explants at 14 WAP in light (a) and dark (b) conditions at 14 WAP

FIGURE 4. Development of embryogenic calluses to form new plants (a) Plantlets in regeneration media 6 months after light treatment and growth regulators; (b) Regenerated young plants aged 20 months; and (c) Flowering *R. gigantea* plants aged 60 months

Conclusions

In this study, the effects of light treatments (light versus dark) and combinations of growth regulators on the induction of somatic embryogenesis in *R. gigantea* were analyzed. The interaction effects of these treatments were also elucidated in this study. Exposure to dark conditions resulted in a higher induction of somatic embryogenesis in *R. gigantea* leaf explants compared to light treatments. Moreover, the addition of 0.5 mg/L^{-1} TDZ plus 0.1 mg/L^{-1} BAP is the optimum combination of growth regulators for the induction of somatic embryogenesis in *R. gigantea*. Meanwhile, in terms of the interaction effect, supplementation of 1.0 mg/L^{-1} TDZ and 0.1 mg/L^{-1} BAP followed by culture incubation under bright light provided optimal conditions for inducing somatic embryogenesis in *R. gigantea*. The findings of this study showed that the optimal environmental conditions (light and hormone formulation) can be repeated to produce *R. gigantea* seedlings in large, uniform quantities for commercialization.

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