SEED GERMINATION AND POLYPLOID INDUCTION USING COLCHICINE IN *Eulophia bicallosa* (D.Don) P.F.Hunt & Summerh.

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การงอกของเมล็ดและการชักนำให้เกิดโพลีพลอยด์โดยใช้สารโคลชิซินในว่านอึ่งอ่าง *Eulophia bicallosa* (D.Don) P.F.Hunt & Summerh.

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ว่านอึ่งอ่าง *(Eulophia bicallosa* (D.Don) P.F.Hunt & Summerh.) เป็นกล้วยไม้ดินที่มีดอกสีเขียวอ่อนหรือสีครีมแต้มด้วยจุดสีม่วง ปัจจุบันจัดอยู่ในกลุ่มกล้วยไม้ใกล้ถูกคุกคามในประเทศออสเตรเลีย ส่วนในประเทศไทย จัดว่าเป็นพืชหายากเนื่องจากพบประชากรเพียงสองกลุ่มเท่านั้น จุดประสงค์ในการศึกษาครั้งนี้เพื่อ 1) ศึกษาผลของความเข้มข้นและระยะเวลาในการฟอกเมล็ดในสารละลายโซเดียมไฮโปคลอไรท์ต่อการมีชีวิตและการงอกของเมล็ด 2) ศึกษาผลของอาหารเพาะเลี้ยงชนิดต่าง ๆ ต่อการงอกและการเจริญเติบโตของเมล็ด และ 3) ศึกษาผลของความเข้มข้นและระยะเวลาในการให้สารโคลชิซินต่อการกระตุ้นให้เกิดโพลีพลอยด์ในว่านอึ่งอ่าง ในการศึกษาผลของสารละลายโซเดียมไฮโปคลอไรท์ต่อการมีชีวิตและการงอกของเมล็ด ผู้วิจัยได้ทำการทดลองโดยการฟอกเมล็ดในสารละลายโซเดียมไฮโปคลอไรท์ที่ความเข้มข้น 0%, 0.5% และ 1% (น้ำหนัก/ปริมาตร) เป็นระยะเวลา 1 5 และ 10 นาที จากนั้นแบ่งเมล็ดที่ผ่านการฟอกเป็นสองส่วน ส่วนหนึ่งนำไปทดสอบร้อยละการมีชีวิตด้วยสารละลายทีทีซีเข้มข้น 1% (1% TTC) อีกส่วนนำไปทดสอบการงอกโดยนำไปเพาะบนอาหารเพาะเลี้ยงสูตร VW เป็นเวลา 4 สัปดาห์ จากนั้นทำการเลือกชุดทดลองที่ดีที่สุดจากร้อยละการมีชีวิตของเมล็ด ร้อยละการงอกของเมล็ด และความสามารถในการควบคุมการปนเปื้อน ในการศึกษาผลของอาหารเพาะเลี้ยงชนิดต่าง ๆ ต่อการงอกและการเจริญเติบโตของเมล็ด ผู้วิจัยได้ทำการทดลองเพาะเมล็ดลงบนอาหารเพาะเลี้ยง 6 ชนิดได้แก่ MM, ½ MM, MS, ½ MS, VW และ ½ VW จากนั้นทำการเก็บผลร้อยละของการงอกและร้อยละของโปรโตคอร์มในระยะต่าง ๆ ทุกเดือนเป็นเวลา 3 เดือน แล้วทำการเลือกอาหารเพาะเลี้ยงที่ดีที่สุดจากสูตรอาหารที่ให้ผลร้อยละการงอกและโปรโตคอร์มที่ระยะสูงสุด ในการศึกษาผลของสารโคลชิซินต่อการเจริญเติบโตและปริมาณดีเอ็นเอ ผู้วิจัยได้นำโปรโตคอร์มอายุ 5 เดือนไปแช่ในสารละลายโคลชิซินความเข้มข้น 0%, 0.025%, 0.05% และ 0.1% (น้ำหนัก/ปริมาตร) นาน 24 ชั่วโมง จากนั้นนำไปเพาะเลี้ยงต่อบนอาหารสูตร MS เป็นเวลา 3 เดือน จดบันทึกพัฒนาการของโปรโตคอร์ม จำนวนยอดต่อโปรโตคอร์ม ความยาวของยอดและราก ร้อยละการมีชีวิต และเมื่อครบ 3 เดือนทำการวัดปริมาณดีเอ็นเอด้วยเครื่องโฟไซโตมิเตอร์ วัดขนาดความกว้างความยาวของปากใบ นับจำนวนเซลล์ผิวใบและจำนวนปากใบต่อพื้นที่ ผลการทดลองพบว่า เมล็ดที่ผ่านการฟอกแล้วนำไปเพาะ

บนอาหารสูตร VW เป็นเวลา 1 เดือน พบว่าเมล็ดที่ผ่านการฟอกในสารละลายโซเดียมไฮโปคลอไรท์ที่ความเข้มข้น 0.5% นาน 5 หรือ 10 นาที ให้ผลร้อยละการรอดชีวิตที่ 62.06 และ 59.10 และให้ผลร้อยละการงอกที่ 58.89 และ 58.42 ตามลำดับโดยไม่มีการปนเปื้อน ส่วนเมล็ดที่ผ่านการฟอกด้วยสารละลายโซเดียมไฮโปคลอไรท์ที่ความเข้มข้น 0.5% หรือ 1.0% นาน 1 นาที ให้ผลร้อยละการรอดชีวิตและการงอกที่สูงกว่า 2 ชุดทดลองที่กล่าวไปแต่มีโอกาสเกิดการปนเปื้อนที่สูงกว่า ส่วนเมล็ดที่ผ่านการฟอกด้วยสารละลายโซเดียมไฮโปคลอไรท์ที่ความเข้มข้น 1.0% นาน 5 หรือ 10 นาทีส่งผลให้ร้อยละการรอดชีวิตและการงอกลดลง ในการศึกษาครั้งนี้ได้ตรวจพบโปรโตคอร์มที่ระยะพัฒนาการต่าง ๆ ดังนี้ ระยะ 1 เอ็มบริโอเกิดการบวม ระยะ 2 เอ็มบริโอขยายขนาดมากขึ้นจนเปลือกเมล็ดแตกออกและระยะ 3 เอ็มบริโอเปลี่ยนเป็นสีเขียวและขยายขนาดขึ้นเป็นอย่างมาก จากการทดลองผลของอาหารเพาะเลี้ยงชนิดต่าง ๆ ต่อการงอกและเจริญเติบโตของเมล็ดพบว่า อาหารเพาะเลี้ยงสูตร MS และ ½ MS ให้ผลการงอกสูงสุดคือ 92.88 ± 7.02% และ 89.21 ± 2.41% และให้โปรโตคอร์มระยะที่สามสูงสุดคือ 91.40 ± 8.60% และ 89.21 ± 2.41% ตามลำดับ รองลงมาเป็นอาหารเพาะเลี้ยง MM½, MM, VW และ ½VW ที่ให้ผลร้อยละการงอกและโปรโตคอร์มระยะที่สามตามลำดับ สำหรับผลของโคลชิซิน พบว่าที่ความเข้มข้น 0.025% และ 0.05% โคลชิซินไม่ส่งผลต่อร้อยละการรอดชีวิตและพัฒนาการของโปรโตคอร์ม ส่วนโปรโตคอร์มที่ได้รับสารโคลชิซินที่ความเข้มข้น 0.1% พบว่ามีการลดลงของร้อยละการรอดชีวิตเล็กน้อยเหลือ 88.33 พืชที่ได้รับโคลชิซินที่ความเข้มข้น 0.05% มีการพัฒนายอดและรากดีที่สุดในเดือนที่ 3 นับหลังจากการได้รับสารโคลชิซิน โดยพบว่าพืชในชุดทดลองนี้ ให้จำนวนยอดต่อต้นเฉลี่ยสูงสุด (1.24 ยอด) ให้ความยาวของยอดเฉลี่ยสูงสุด (4.15 ซม.) ให้ร้อยละการชักนำยอดเฉลี่ยสูงสุด (35.33%) ให้ความยาวของรากเฉลี่ยสูงสุด (2.51 ซม.) ให้ร้อยละการชักนำรากเฉลี่ยสูงสุด (21.67%) นอกจากนี้ยังพบการขยายตัวของปากใบและเซลล์ผิวในพืชที่ได้รับโคลชิซิน โดยพบว่าที่ความเข้มข้น 0.025% พืชมีจำนวนเซลล์ผิวใบต่อพื้นที่เฉลี่ยที่ 43.78 เซลล์ต่อ 0.1 mm^2 ซึ่งน้อยกว่าชุดควบคุมอย่างมีนัยสำคัญ และที่ความเข้มข้น 0.05% พืชมีขนาดความยาวปากใบเฉลี่ยที่ 36.32 ไมโครเมตร และกว้างเฉลี่ยที่ 30.41 ไมโครเมตร ซึ่งใหญ่กว่าชุดควบคุมอย่างมีนัยสำคัญ ในส่วนของปริมาณดีเอ็นเอพบว่ามีการเพิ่มขึ้นในพืชที่ได้รับโคลชิซินที่ความเข้มข้น 0.05% และ 0.1% โดยมีการพบกลุ่มประชากรของเซลล์ที่มีปริมาณดีเอ็นเออยู่ประมาณ 4C-8C เทียบกับพืชที่ไม่ได้รับโคลชิซิน (ชุดควบคุม) ซึ่งพบปริมาณดีเอ็นเอ 2C, 2C-4C และ 4C

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ปีการศึกษา 2566 ลายมือชื่ออาจารย์ที่ปรึกษา

WORAPHAT KHANKHOKKRUAD : SEED GERMINATION AND POLYPLOID INDUCTION USING COLCHICINE IN *Eulophia bicallosa* (D.Don) P.F.Hunt & Summerh. THESIS ADVISOR : ASSOCIATE PROFESSOR NOODUAN MUANGSAN, Ph.D. 128 PP.

Keywords: orchid seed germination, polyploid induction, *Eulophia bicallosa*, colchicine, sodium hypochlorite, seed viability test

Eulophia biocallosa (D.Don), a terrestrial orchid having flowers that are light green or cream in color flowers with purplish markings, is considered a near-threatened species in Australia. It is also recognized as a rare species in Thailand since just two populations have been reported so far. The objectives of this thesis were to 1) investigate the effect of various Na(OCl) concentrations and durations on viability and seed germination, 2) investigate the effect of various media on seed germination and growth of *E. bicallosa* s' seeds, and 3) investigate the effect of colchicine concentration and duration on polyploid induction of *E. bicallosa*. The seeds were treated with Na(OCl) at three concentrations (0.0, 0.5, and 1.0% (w/v)) and three durations (1, 5, and 10 min). After that, 1% TTC was employed for the viability test. Sterilized seeds were then cultured on Vacin and Went (VW) media for four weeks. The optimal condition was chosen based on the percentage of seed viability, seed germination, and a presence of contamination. To investigate the effect of various media on seed germination and growth, seeds of *E. bicallosa* were cultured on six different types of media (MM, ½ MM, MS, ½ MS, VW, and ½ VW). Seed germination and protocorm development were recorded monthly for 3 months, and the optimal medium was chosen based on the percentage of seed germination and the most advanced seed development stage. To investigate the effect of colchicine on orchid plants, 5-month-old protocorms were treated with four different concentrations of colchicine (0, 0.025, 0.05, and 0.1 (w/v)) for 24 hours and continue cultured on MS medium. The protocorm development, the number of shoots per protocorm, the length of root and shoot, and the survival rate were examined monthly for 3 months. The length and width of stomata, the number of stomata and leaf epidermal cells, and the DNA content were also examined using flow cytometry in the 3rd month of culture. The result revealed that, after four weeks of sowing, seed treatments with 0.5% (w/v) Na(OCl) for 5

min and 0.5% (w/v) Na(OCl) for 10 min resulted in high seed viability of 62.06% and 59.10%, respectively, along with seed germination rates of 58.89% and 58.42%, without any contamination. However, seed treatments with 0.5% or 1% (w/v) Na(OCl) concentrations for 1 min resulted in increased seed germination and seed viability but also resulted in contamination. Prolonged treatments (5 min or 10 min) with 1% (w/v) of Na(OCl) caused a decline in seed viability and germination. The development of *E. bicallosa* seeds was observed through 3 Stages including Stage 1; embryo swelling, Stage 2; embryo getting bigger and rupture of the testa, and Stage 3; where the embryo turned green. For the effect of various media on seed germination and growth, MS and ½MS gave the highest percentage of seed germination with 92.88 ± 7.02% and 89.21 ± 2.41%, respectively, and the highest percentage of Stage 3 development seeds with 91.40 ± 8.60% and 89.21 ± 2.41 respectively followed by MM, ½MM, VW, and ½VW media. For the effect of colchicine treatments, 0.025% and 0.05% colchicine treatments did not affect the survival rate and protocorm development. However, a slight decrease in the survival rate to 88.33% was observed in the 0.1% colchicine treatment. Plants treated with 0.05% colchicine exhibited the greatest shoot and development in the third month after treatment, with the highest average shoots per plant (1.24 shoots per plant), average length of shoot (4.15 cm), average shoot induction percentage (35.33%), average length of root (2.51 cm), and average root induction percentage (21.67%). The size of stomata and leaf epidermal cells also increased due to the effect of colchicine. Plants treated with 0.025% colchicine had a significantly lower number of leaf epidermal cells per area (43.78 cells per 0.1 mm^2) than plants in the control treatment, and plants treated with 0.05% colchicine had significantly longer stomatal length (36.32 µm) and width (30.41 µm)For the effect of colchicine on DNA content, plants treated with 0.05% and 0.1% colchicine possessed a population of cells with 4C-8C DNA content, while non-colchicine treatment cells (control) possessed 2C, 2C-4C, and 4C DNA content.

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Woraphat Khankhokkruad

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LIST OF ABBREVIATIONS

Abbreviation	Definition
TTC	Triphenyl tetrazolium chloride
VW	Vacin and Went
½VW	Half strength Vacin and Went
MM	Malmgren
½MM	Half strength Malmgren
MS	Murashige and Skoog
½MS	Half-strength Murashige and Skoog
µg	Microgram
g	gram
µm	Micrometer
µl	Microliter
L	liter
µM	Micromolar
cm	Centimeter
ml	Milliliter
cm^2	Centimeter square
mm^2	Millimeter square
% (w/v)	Percent weight by volume
ซม.	เซนติเมตร

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Orchidaceae (orchids) is a family of herbaceous plants that can be found on trees, rocks, and soil. They have a unique and beautiful flower that possesses a highly modified dorsal petal (lip) (Taylor, Taylor, and Krings, 2009), making them have a high market need and causing them to be removed from natural habitats. Some orchid seeds are hard to germinate and have a high mortality rate (Kauth et al., 2014), and now many species are at risk of extinction.

Eulophia bicallosa (D.Don) P.F.Hunt & Summerh. Is a terrestrial orchid that possesses beautiful white purplish flowers. The flowering period occurs during April and December. It can be found in southeast Asia (India, Indonesia, Malaysia, Myanmar, Nepal, New Guinea, Thailand, and Australia) (Chen et al., 2009). It has a high potential as a commercial plant that has become more popular in local markets due to its beautiful flowers.

People believe that this orchid species has medicinal properties and can bring good fortune, thus making it popularly sold through many online platforms such as YouTube, Facebook, Lazada, and Shopee. These situations raise a concern that this orchid species may become a threatened species in the near future (Narkhede et al., 2016).

Each *Eulophia* species needs different conditions for growth and germination, even though they are reported to germinate in many media and environmental conditions. Culturing *Eulophia* seeds in improper conditions may lead to abnormal development (Decruse et al., 2013), growth stunning and failure to develop into seedlings (Nanekar et al., 2014), and failure to germinate (McAlister and Staden, 1998). Among many reports, MS (Murashige and Skoog, 1962), VW (Vacin and Went, 1949), and MM (Malmgren, 1996) are the most successful media. MS alone was claimed to give a high germination rate (70-80%) (McAlister and Staden, 1998), while MS supplemented with 10% (v/v) coconut water yielded the biggest protocorm when compared to Mitra and Knudson-C media (Decruse et al., 2013). MM has the highest number of Stage 2 protocorm within 18 weeks of culture when compared with Knudson-C, P723, ½ MS, and VW media. VW gave the most advanced protocorm (Stage 4) at the highest number (1.3%) (Johnson, 2007). However, it has been not yet reported on culture media effects on seed germination of *E. bicallosa*.

There are many ways to produce new varieties in orchids for ornamental use, such as crossbreeding, selection-breeding, mutation induction, molecular marker-assisted breeding, gene transfer, and genome editing (Li et al., 2021). Among these methods, polyploid induction (one of the mutation induction methods) is the most convenient approach, because it does not require high-level knowledge, and polyploid plants usually contain unique traits that are different from natural varieties. When compared to natural diploid varieties, polyploid orchids could have a change in leaf shape and color (Li et al., 2021), plant height and flower size (Chen and Tang, 2018), or secondary metabolite content (Chung et al., 2017).

Polyploid induction is the method for changing a normal organism (all of the cells contain two sets of chromosomes) into a polyploid organism (most or all of the cells contain more than two sets of chromosomes). Plants can be made polyploid in two ways: 1) physically induced polyploid, and 2) chemically induced polyploid. Physically induced polyploidy creates polyploid plants by cutting explant parts with an abundance of polyploid cells and culturing them on a medium, resulting in polyploid plants. This method is limited to some species and requires polyploid cell distribution knowledge that varies by species (Chen and Tang, 2018). For chemical-induced polyploids, this method creates polyploid plants by treating explants with antimitotic reagents at optimal concentration and duration. The antimitotic reagent will interrupt cell division, resulting in one daughter cell that contains all of the chromosomes (Van Vuuren et al., 2015). There are many attempts to induce polyploidy orchids with colchicine. The application of colchicine for polyploid induction in orchid development is an important method for plant development to increase the size of the flowers, pseudobulbs, or other desired traits. The commonly used concentrations of colchicine are 0.01–1.0% (w/v) and vary by species. Even though colchicine treatment has been a popular method for polyploid induction in many plants, no polyploid plant has been induced in *E. bicallosa.*

Therefore, the objectives of this study were to investigate 1) the effect of Na(OCl) concentration and duration time on seed viability and germination, 2) the effect of various media on seed germination and protocorm development of *E. bicallosa*, and 3) the effect of colchicine (as an antimitotic reagent) concentration and duration time on polyploid induction of *E. bicallosa*. It was hypothesized that the optimal Na(OCl) concentration should have a reasonable contamination control and minimal effect on seed viability and germination of *E. bicallosa*, the suitable medium might better help to promote seed germination and development of *E. bicallosa* than other media, and colchicine treatment might be effective in inducing polyploidy in *E. bicallosa* protocorms. The effects of colchicine on plant growth, DNA content, and chromosome number of treated plants were also examined.

1.2 Research objectives

The main objective of this work was to develop a protocol for polyploid induction of *E. bicallosa.* Specific objectives are described below.

1.2.1 To investigate the effects of Na(OCl) concentration and treatment duration on viability, germination, and sterilization of *E. bicallosa* 's seeds.

1.2.2 To investigate the effects of culture media on seed germination and protocorm development of *E. bicallosa* 's seed.

1.2.3 To investigate the effects of colchicine concentration and duration time on polyploid induction of *E. bicallosa*

1.3 Research hypothesis

1.3.1 The optimal Na(OCl) treatment should have reasonable contamination control and minimal effect on seed viability and germination.

1.3.2 The suitable medium might better help to promote seed germination and protocorm development of *E. bicallosa.*

1.3.3 Colchicine treatment might be effective in inducing polyploidy in *E. bicallosa* protocorms.

1.4 Scope and limitations of the study

This study only focused on studying the effect of Na(OCl) concentrations and duration treatment on seed viability and germination of *E. bicallosa.* To study the effect of various media on seed germination and protocorm development, only six types of media (MS, MM, ½MS, ½MM, VW, and ½VW) were used, and the suitable media was determined based on the percent of seed development and highest seed development stage. To study the effect of colchicine on orchid plants, *E. bicallosa* was treated with various concentrations of colchicine for 24 hours. After that, DNA content, plant growth, and leaf anatomy were compared among treatments.

1.5 Expected results

1.5.1 Obtain the optimal Na(OCl) treatment with reasonable contamination control and low effect on seed viability and germination

1.5.2 Obtain a suitable medium that can promote seed germination and protocorm development.

1.5.3 Obtain the optimal colchicine treatment for polyploid induction that can affect plant growth and DNA content.

CHAPTER II

LITERATURE REVIEW

2.1 Orchidaceae

Orchidaceae is an herbaceous plant family, usually perennial but sometimes annual. They can be found on a variety of surfaces, including trees (epiphyte), rocks (lithophyte), and soil (terrestrial). They possess modified stems in the form of rhizomes or tubers. Their roots are infected with mycorrhiza or modified as photosynthesis aerial roots. Photosynthesis aerial root possesses one or more velamen layers (dead cell layers) (Chen et al., 2009). Their flowers vary in size and are usually zygomorphic (rarely actinomorphic) (Chen et al., 2009). A flower has three sepals, three petals, and one column. One of the three petals is highly modified and usually ornamented with an appendage, spur, or nectary, as well as hair usually called lip or labellum (Li et al., 2021). Now the orchid family contains 800 genera, more than 28,000 species, that can be found all over the world, especially in tropical and sub-tropical areas (194 genera), but not in Antarctica (Chen et al., 2009). These plants have usually high market value, due to their rarity, beautiful ornamental plants (Li et al., 2021), and medicinal properties (Chung et al., 2017).

2.2 *Eulophia* characteristic

Eulophia is a genus in orchidaceae family. Plants in this group are typically found on land, autotrophic, some of them are heteromycotrophic. They have underground structures called pseudobulbs which sometimes can be found above ground. Pseudobulbs have many form including corm-like, tuberous, or rhizomatous, usually possess several nodes and several thin or thick fibrous roots at their base. Their leaves appear either during or after the flowering period, and they can range from one to many, generally located at the plant's base and may have a tapered or contracted base that resembles a long, stem-like stalk. In heteromycotrophic species, these leaves may be reduced to scales. Sometimes, the petiole-like leaf base can overlap and create a false stem. The inflorescence stands upright and can be found on the side of the plant. It is typically arranged in a linear pattern, occasionally branching out into a loose or somewhat densely packed arrangement with many flowers, but sometimes it consists of just a single flower. The flowers themselves can vary in size and are sometimes quite attractive. Pedicel and ovary are slender. Each sepals are separate from each other and look similar. The lateral sepals are usually slightly slanted and may be partially attached to the foot of the central column of the flower. The petals closely resemble to dorsal sepal or are slightly wider. The lip is typically erect, has three lobes, lateral lobes erect and encircle the central column. Sometimes lip may be whole, and its base often forms a spur or sac-like structure. The disk typically contains a callus made up of layers, ridges, or fringed structures. The column is varies in length, usually winged, may or may not have a column foot. The anther is positioned at the tip of the column, incompletely 2-locular, anther cap has two protrusions. Two waxy pollinia attached to a suborbicular viscidium by a wide stalk short (Chen, Cribb and Gale. 2009).

2.3 *Eulophia bicallosa* (D.Don) P.F.Hunt & Summerh.

E. bicallosa is an autotrophic terrestrial orchid, 50-75 cm in height. It has a pseudobulb that lies below the soil surface, is white, subglobose to cylindric shape, 5-10 cm in size, and is covered with sheaths. Leaf will emerge after the flowering season, 50 cm long and 1.2 cm wide, with linear shape, acute or acuminate apex. The inflorescence is a slender racemose shape, 50-75 cm long, covered with 3-5 tubular sheath, 10 cm rachis, and composed of few to many flowers. Floral bract is lanceolate in shape, 8-14 mm in size, and has an acuminate apex. The flower is 25 mm diameter in size. Three sepals are pale green, 5 purple veins, lanceolate shape, ca. 13 mm long and 3-5 mm wide, and have acute apex. The lateral sepal attaches to the column foot. The lateral petals are pale green, tingled with purplish red at the apex, lanceolate to elliptic shape, 13 mm long and 7-8 mm wide, and have acute apex. The lip is subovate in shape, 15 in mm size, and composed of 3 lobes. The lateral lobes are erect and triangle-shaped. The midlobe is a curved ovate shape, retuse apex. The spur is saccate, 4 mm, obtuse apex. The column is 6 mm in size, slander, and has a distinct column foot (Chen et al., 2009). This species can be found native to many countries in southeast Asia (Figure 2.1).

Figure 2.1 *E. bicallosa* 's native country: Assam (India), Bangladesh, Bismarck Archipelago, East Himalaya, Hainan, Jawa, Malaya, Nepal, New Guinea, Northern Territory, Philippines, Queensland (Australia), Sumatera, Thailand, West Himalaya, Western Australia (© Copyright 2023 International Plant Names Index and World Checklist of Vascular Plants. http://creativecommons.org/licenses/by/3.0)

E. bicallosa is believed to have medicinal properties. Some people in India consume its tuber as food and medicine. (Narkhede et al., 2016). Its extract contained flavonoid 0.08 ± 0.004 mg/g of quercetin equivalent, and proanthocyanidin 0.16 ± 0.038 mg/g of catechin equivalent. It is also proved that its extract has free radical scavenging activity by using FRAP assay (0.05±0.001 μM Fe (II)/g of dry mass) and NO scavenging assay (15.32% inhibition) (Narkhede et al., 2016). Now this species is considered as near threaten species in Australia (Department of Environment and Science, Queensland, 2013) and endangered species in India (Baro, Bawri, Adhikari, and Borthakur, 2019).

2.4 *Eulophia* seed germination

Eulophia species need different conditions for growth and germination depending on species variation, even though they are reported to germinate in many media and environmental conditions. Culturing *Eulophia* seeds in improper conditions may lead to abnormal development (Decruse et al., 2013), growth stunning and failure to develop into seedlings (Nanekar et al., 2014), and failure to germinate (McAlister and Staden, 1998). Among many reports, MS (Murashige and Skoog, 1962), VW (Vacin and Went, 1949), and MM (Malmgren, 1996) are the most successful medium. MS alone was claimed to give a high germination rate (70-80%) (McAlister and Staden, 1998), while MS supplemented with 10% coconut water yielded the biggest protocorm when compared to Mitra and Knudson-C media (Decruse et al., 2013). MM had the highest number of stage 2 protocorm within 18 weeks of culture when compared with Knudson-C, P723, 1/2MS, and VW media. VW gave the most advanced protocorm (stage 4) at the highest number (1.3%) (Johnson, 2007).

2.5 Polyploid induction

Polyploid is a phenomenon where some or most of the cells of one organism contain more than two sets of chromosomes. In orchids, polyploidization makes them acquire unique traits that are different from natural varieties, such as a change in leaf shape and color (Li et al., 2021), plant height and flower size (Chen and Tang, 2018), or secondary metabolite content (Chung et al., 2017).

Polyploids can occur naturally or through artificial induction. For artificial polyploid induction in plants, there are two ways to do it; 1) physically induced polyploid, and 2) chemically induced polyploid. Physical-induced polyploid plants are created by cutting explant parts with an abundance of polyploid cells and culturing them on a medium, resulting in polyploid plants. This method is limited to some species and requires polyploid cell distribution knowledge that is different among species (Chen and Tang, 2018). Chemical-induced polyploid plants are created by treating normal plants or polyploid plants (to increase ploidy levels) with antimitotic reagents in optimal

concentration and duration. The antimitotic reagent will interrupt cell division, resulting in one daughter cell that contains all of the chromosomes. Several types of anti-mitotic reagents are reported to be used in plant polyploid induction such as colchicine, oryzalin, trifluralin, flufenacet, and a mixture of amiprophos-methyl + pronamide + dimethyl sulfoxide. Among these reagents, colchicine is the most widely used and successful in many orchid species (Niazian and Nalousi, 2020) as seen in Table 2.1.

Colchicine is an alkaloid compound that is extracted from *Colchicum autumnale* L. It acts as an anti-mitotic-reagent by binding to B-tubulin in microtubules during metaphase, destabilizing, and depolymerizing microtubules, preventing microtubules from attaching to chromosomes and interrupting chromosome separation during anaphase (Eng and Ho, 2019). As a result, two sets of chromosomes remain in one nucleus, and polyploid cells are born after the cell cycle ends. Since 1550 BCE, it has been used in medicine to cure gout attacks (Eng and Ho, 2019). In plants, it was first used for *in vivo* polyploid induction by applying to plant tissue through immersion of protocorm in colchicine solution (Zhang and Gao, 2020), wrapping colchicine-soaked-cotton around the ovary (Azmi et al., 2016), or culture adventitious-shoot on colchicine-supplemented-medium (Huy et al., 2019). For *in vitro* polyploid induction, the first report since 1966 by Murashige and Nakano, who conducted *in vitro* polyploid induction on tobacco and succeeded in producing polyploid tobacco calli (Sattler, Carvalho, and Clarindo, 2015).

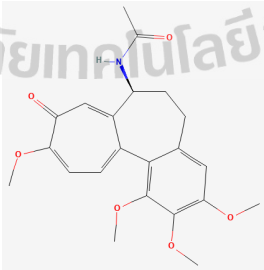

Figure 2.2 Colchicine structure.

(https://pubchem.ncbi.nlm.nih.gov/compound/Colchicine#section=Structures)

Table 2.1 Effects of colchicine concentrations, treating duration and treating methods on growth and morphology of Orchidaceous species.

Species	Explant	Treating method	Concentration	Duration (hours)	Result	Reference
Dendrobium chrysotoxum Lindl.	Protocorm	Culture on semi-solid VW medium supplemented with colchicine	0, 0.01, 0.02, 0.03, 0.04, 0.05 % (w/v)	24, 48, 72, 96, 120	Shoot proliferation stimulated	Atichart, 2013
Phalaenopsis amabilis (L.) Blume.	Pollinated Flowers	Wrap colchicine-soaked cotton around the ovary	50, 500 mg/L	72, 120	Germination: inhibited Leaf: smaller	Azmi et al., 2016
Paphiopedilum villosum (Lindl.) Stein.	Adventitious Shoot	Culture in SH mediums supplemented with colchicine in the dark	2, 10, 50 μM	72, 144, 216	Of all treatments, 84 % are tetraploid, and the rest are mixoploid	Huy et al., 2019

Table 2.1. Effects of colchicine concentrations, treating duration, and treating methods on growth and morphology of Orchidaceous species (Continued).

Species	Explant	Treating method	Concentration	Duration (hours)	Result	Reference
Dendrobium cariniferum Rchb.f.	Protocorm	Immerse in autoclaved colchicine solution in the dark	0.03, 0.05, 0.1 % (w/v)	18, 24, 36, 48	Plant height: shorten. Leaf: greener, fleshier, broader and shorter, lower leaf trichome density Stem: greater stem. Root: diameter increased, shortened Trichome: color changing, Stomata: lower cell and stomata density, bigger	Zhang and Gao, 2020
Dendrobium santana x *D. friedericksianum*	Nodal segment	Treat nodal segment from seedling with colchicine	0.01–0.1 g/v	24 - 72	DNA content: increased. 33-50 % Guard cell: bigger, lower density, get more chloroplast	Choopeng, Te-chato and Khawnium, 2019

2.6 Detection and characterization of polyploid plants

There are two ways to detect polyploid plants, indirect and direct methods (Sattler, Carvalho and Clarindo, 2015). Indirect methods detect morphological and physiological changes such as leaf shape (Azmi et al., 2016), growth rate and plant height (Zhang and Gao, 2020), secondary metabolite content (Chung et al., 2017), and stoma size (Azmi et al., 2016). These parameters are usually easy to measure but are inaccurate due to a change in these parameters that may be caused by factors other than genome duplication (polyploid) such as gene mutation or environmental factors. As a result, indirect methods need to be confirmed with the direct methods that directly detect changes in cell DNA content. There are two ways to measure cells' DNA content, flow cytometry technique, and chromosome counting technique. Flow cytometry is a technique for determining the DNA content of many cells simultaneously. Even though this technique is rapid and reliable, it is also quite complicated and needs to be operated with care, since many factors can cause errors in the result such as tissue sample, dye, buffer, and fixation technique (Darzynkiewicz and Juan, 1997). Therefore, the chromosome-counting technique may be needed to confirm that polyploid cells exist in the tissue.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Explant source and seed storage

For the seed germination test experiment, *Eulophia bicallosa* pods and seeds were collected from Rayong Botanical Garden, Rayong province with a permit. Then, seed capsules were stored in paper bags and dried on silica gel until dehiscence. The brown seeds were then stored in a sterile Eppendorf tube at 4 °C (12% humidity) until use. For the polyploid induction experiment, *E. bicallosa* protocorms at stages 3-5 cultured on a suitable medium for 3-4 months were used for all treatments.

3.2 Seed sterilization treatments

3.2.1 Prepared 28 sets of 2 mg seeds in a 1.5 ml Eppendorf tube (4 tubes per treatment).

3.2.2 Added 500 µl of three Na(OCl) concentrations (0.0%, 0.5%, and 1.0% (w/v)) to each tube using a micropipette with 1,000 µl tip, alternating between inverting the tube back and forth for 30 sec and resting for 30 sec for a set duration (1 min, 5 min, and 10 min). All treatments were treated for three duration times (1 min, 5 min, and 10 min) except 0% Na(OCl) concentrations only treated for 10 min.

3.2.3 Used a micropipette with 100 µl tip to remove Na(OCl) solution as much as possible.

3.2.4 Added 1,000 µl DI water and alternated between inverting the tube back and forth for 30 sec and resting for 30 sec for 5 min. Used a micropipette with 100 µl tip to remove DI water as much as possible. This step was repeated 3 times.

3.2.5 Treated seeds were suspended in 500 µl DI water. Pipetted 500 µl seed suspension into a new 1.5 ml Eppendorf tube, using a micropipette with a modified 1000 µl tip. Each tube was used for viability and germination tests.

3.2.6 The viability of treated seeds was tested by removing DI water from seed suspension as much as possible, using a micropipette with a 100 µl tip. After that, applied 2-3 drops of 1% triphenyl tetrazolium chloride (TTC), and incubated at 30 ± 2 °C for 2 days. Embryos that are orange or reddish were considered viable, then the seed viability percentage was calculated by using the formula below. Each treatment contains 4 replicates, and each replicate contains 150 – 200 seeds.

$$\text{viability percentage} = 100 \text{ x} \frac{\text{number of viable seed}}{\text{number of total seed}}$$

3.2.7 The germination of treated seeds was tested by removing DI water from seed suspension as much as possible, using a micropipette with a 100 µl tip. After that suspended seeds in 150 µl 0.08% (w/v) agar, pipette seed suspension using a micropipette with a modified 1000 µl tip to disperse on a plate containing 20 ml VW (Vacin and Went, 1949) culture medium, solidified with 0.6% (w/v) agar, cultured for one month, under darkness, 25 ± 2 °C. The seeds that developed to Stage 1 protocorm or higher (Stewart and Zettler, 2002) (Figure 3.1) were considered germinated seeds, and then the seed germination percentage was calculated by using the formula below. Each treatment contains 4 replicates, and each replicate contains 150 – 200 seeds.

$$\textbf{germination percentage} = \textbf{100x} \frac{\textbf{number of germinated seed}}{\textbf{number of total seed}}$$

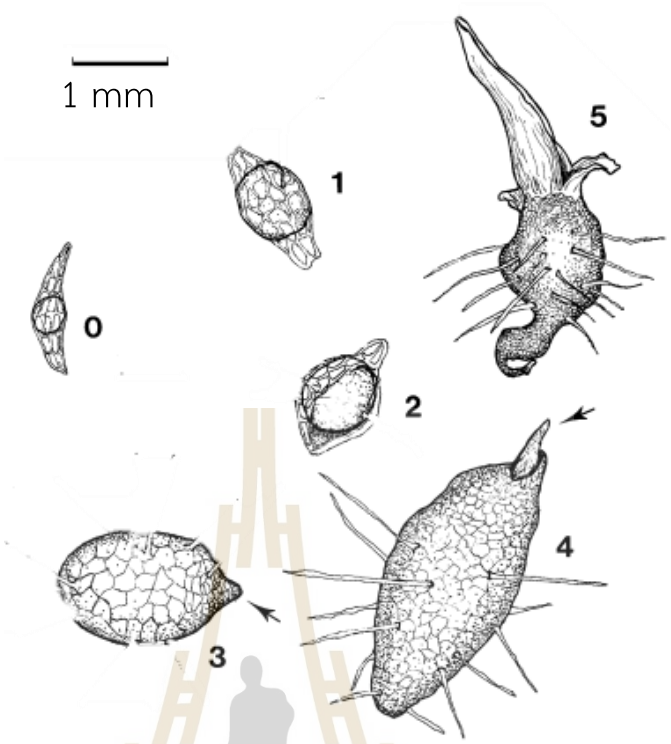

Figure 3.1 Orchid seed development. Adapted from Stewart and Zettler (2002) and Stewart and Kane (2006). Stage 0: not germinated seed, Stage 1: Seed swelling due to water imbibition and cell division, Stage 2: embryo getting bigger and rupture of testa due to cell division, Stage 3: embryo greening enlargement due to chlorophyll synthesis and cell division, Stage 4: appearance shoot or root, Stage 5: the appearance of first leaf.

3.3 Seed culture experiment

3.3.1 Prepared 6 sets of 3 mg seeds in a 1.5 ml Eppendorf tube (1 tube per treatment), sterilized using a method and optimal Na(OCl) condition from part 3.2.

3.3.2 Sterilized seeds were suspended on 1000 µl 0.08% agar and pipetted for 200 µl using a micropipette with a modified 1000 µl tip to disperse on a plate containing 20 ml culture medium. There are six different types of media used in this experiment including 1) MS (Murashige and Skoog Basal Medium with FeNa-EDTA) (Murashige and Skoog, 1962), 2) 1/2MS, 3) MM (Malmgren Modified Terrestrial Orchid Medium Without Sucrose and Agar) (Malmgren, 1996), 4) 1/2MM, 5) VW (Vacin and Went, 1949), and 6) ½ VW. The details of each medium are in Table 3.1. Seeds were cultured for 3 months, under darkness (first 4 weeks) and under 16 hours photoperiod (next 8 weeks), 25 ± 2 °C. Seed germination and protocorm development were recorded according to Figure 3.1 (Stewart and Zettler, 2002) every month. Then seed germination percentage and protocorm development percentage of each stage were calculated by using the germination percentage formula and formula below: * 150-200 seeds/plate, 5 plates/treatment.

$$\textbf{protocorm development stage percentage} = \textbf{100x}\frac{\textbf{number of stage x protocorm}}{\textbf{number of total seed}}$$

Table 3.1 Composition of MS, MM and VW media.

Component	MS (mg/L)	MM (mg/L)	VW (mg/L)
MACROELEMENTS			
Ammonium sulphate $(NH_4)_2SO_4$			500
Ammonium nitrate NH_4NO_3	1650		
Calcium phosphate $Ca_3(PO_4)_2$		75	200
Calcium Chloride ($CaCl_2$)	332.2		
Magnesium sulphate heptahydrate $MgSO_4 * 7H_2O$			250
Magnesium sulphate, $MgSO_4$	180.7	97.69	
Potassium nitrate, KNO_3	1900		525
Potassium phosphate monobasic KH_2PO_4	170	75	250
MICROELEMENTS			
$FeSO_4$. $* 7H_2O$		27.8	27.8
Manganese sulphate tetrahydrate $MnSO_4 * 4H_2O$			7.5
Manganese sulphate monohydrate $MnSO_4 * 1H_2O$	16.9	1.54	
Na_2EDTA			37.3
$Na_2EDTA * 2H_2O$		37.26	
FeNaEDTA	36.7		
Boric acid	6.2		
Cobalt chloride hexahydrate $CoCl_2 * 6H_2O$	0.025		
Copper sulphate pentahydrate (Cupric Sulfate $* 5H_2O$) $CuSO_4 * 5H_2O$	0.025		

Table 3.1 Composition of MS, MM and VW media (Continued).

Component	MS (mg/L)	MM (mg/L)	VW (mg/L)
Molybdic acid (sodium salt)	0.25		
Na_2MoO_4			
Potassium Iodide, KI	0.83		
Zinc sulphate heptahydrate	8.6		
$ZnSO_4$·* $7H_2O$			
VITAMIN			
Biotin		0.05	
Folic acid		0.5	
Myo-Inositol	100	100	
Nicotinic acid (free acid)	0.5	5	
Pyridoxine HCl	0.5	5	
Thiamine hydrochloride	0.1	10	
AMINO ACID			
Glycine	2	2	
OTHER			
Activated charcoal		1000	
Casein hydrolysate		400	
Pineapple powder		20000	
CARBON SOURCE			
Sucrose	30000	10000	20000
pH	5.7-5.8	5.5-6	4.8-5.5
Agar	6000	6000	6000

Remark: Half-strength media: reduce all composition to half except agar.Half-strength media and full-strength use the same Ph range.

3.4 Colchicine treatments

3.4.1 Prepared 4 sets of randomly picked 180 c protocorms at stages 3-5 from suitable medium, part 3.3.

3.4.2 Put the selected protocorms into a vial bottle, containing 2 ml colchicine solution, and leave it under the darkness for 24 hours. There are four colchicine concentrations used in this experiment including 0, 0.025, 0.05, and 0.1 (w/v) (each treatment contains 180 protocorms)

3.4.3 Used a micropipette with 1000 µl tip to remove colchicine solution as much as possible from the vial bottle.

3.4.4 Applied 3000 µl DI water to a vial bottle with treated protocorms, agitating for 30 sec and resting for 30 sec for 5 min, and used a micropipette with 1000 µl tip to remove DI water as much as possible. This step was repeated 3 times.

3.4.5 Dispersed 180 treated protocorms on three 8 oz culture bottles containing 50 ml and three plates containing 20 ml of optimal culture medium from part 3.3. (30 protocorms per culture vessel).

3.4.6 Cultured for 3 months, recorded protocorm development, number of shoots and roots per plant, length of shoot and root, calculated protocorm development of each stage percentage and survival rate, using protocorm development stage x percentage formula and formula below:

$$\textbf{Survival rate} = \textbf{100x}\frac{\textbf{number of living plant}}{\textbf{number of total plant}}$$

3.4.7 Analyze sample using flowcytometry techniques. Containing 5 steps including: 1). Put 0.5-1.0 g of leaf into a petri dish containing 500 µl Quantum Stain NA UV 2 (A), 2) added 0.01-0.05 g Polyvinyl-Pyrrolidone (PVP) (40,000 MW) and chop the leaf into pieces (3 mm length and 3 mm width per piece or smaller), 3) filtrated nuclear suspension through a cell strainer (30 µm diameter pore), 4) added 500 µl Quantum Stain NA UV 2 (B), and incubated for 1 min (25 °C), 5) analyzed samples using a Quantum Analysis Flow cytometer (model: QA FCM QP 2, Germany

3.4.8 preparing leaf sample for anatomical analysis. Containing 6 steps including: 1) Randomly pick 3 normal plants (without hyperhydricity symptom) from the control treatment and randomly pick 3 polyploid suspected plants per treatment (abnormal morphology) (Figure 4.4) from 0.025, 0.05, 0.1 (w/v) colchicine treatments, 2) Choose the biggest leaf of each plant and cut it into pieces (0.4 cm^2 square), 3) Soak the pieces of cut leaf in 95% alcohol for 3 hours or until bleached. 4) Randomly pick 3 pieces of bleached leaf and observe under a microscope, 200x magnification, 5) Randomly pick 3 areas of observation (0.1 mm^2 each) per piece of bleached leaf and record the number of stomata and the number of cells per area of observation, 6) Record 10 stomatal length and width per piece of bleached leaf.

3.5 Experimental design and statistical analysis

Table 3.2 Experimental design and Statistical analysis of each experiment.

Experiment	Parameters	Experimental unit	Replicate	Statistic
3.2. Sterilization Treatment	1. Germination percentage	150-200 seeds/rep	4	ANOVA+LSD (p=0.05)
	2. Viability percentage	150-200 seeds/rep	4	ANOVA+LSD (p=0.05)
3.3. Seed culture experiment	1. Germination percentage	150-200 seeds/rep	5	ANOVA+LSD (p=0.05)
	2. Protocorm development of each stage percentage	150-200 seeds/rep	5	ANOVA+LSD (p=0.05)
3.4 Colchicine treatments	1. Protocorm development percentage	A group of 30 plants	6	ANOVA+LSD (p=0.05)
	2. Survival rate	A group of 30 plants	6	ANOVA+LSD (p=0.05)
	3. number of shoots and roots per plant	A group of 30 plants	6	ANOVA+LSD (p=0.05)
	4. length of the longest shoot and root	A group of 30 plants	6	ANOVA+LSD (p=0.05)
	5. number of stomata and cells	9 observation area ($0.1\ mm^2$ each)/plant	3	ANOVA+DMRT (p=0.05)
	6. stomatal length and width	30 stomata/plant	3	Kruskal-Wallis test (p=0.05)

CHAPTER IV
RESULTS AND DISCUSSION

4.1 The effect of Na(OCl) concentration and treatment duration on viability and germination of *E. bicallosa* 's seed

Embryos of the viable seeds of *E. bicallosa* stained bright red after treatment with 1% TTC for 2 days, this could be used as a criterion to determine viable seed. During the first month of observation, the majority of the seeds were found to be in Stages 0 and Stage 1 (germinated) (Figure 4.1). However, the germination percentage varied among different treatments as shown in Table 4.1. The results in Table 4.1 show that control treatment or original seed batches (0x10) gave a 66.90% average viability percentage. Unfortunately, no data on seed germination was available for this treatment due to contamination in all experimental units. The treatments 0.5x1 and 1x1 gave the highest seed viability (91.95% and 87.37%) and germination percentages (91.70% and 83.52%) but some contamination was observed in these two treatments, possibly because of too low concentration of Na(OCl) or too short duration treatments, resulting some microbes remained in the tissue.

On the other hand, the treatments 1x5 and 1x10 had the lowest seed viability (13.82% and 4.70%) and germination percentages (21.14% and 1.49%). These two treatments might have used a higher concentration of Na(OCl) or treated the seeds for too long, resulting in excessive tissue damage and inhibited germination. The treatments 0.5x5 and 0.5x10 gave average moderate seed viability (62.06% and 59.10%, and germination percentages (58.89% and 58.42%) without any contamination issues. Therefore, these two treatments were chosen as the optimal conditions because they had good contamination control which is essential for the long-term culture period of orchids required a long time to develop from seeds to seedlings.

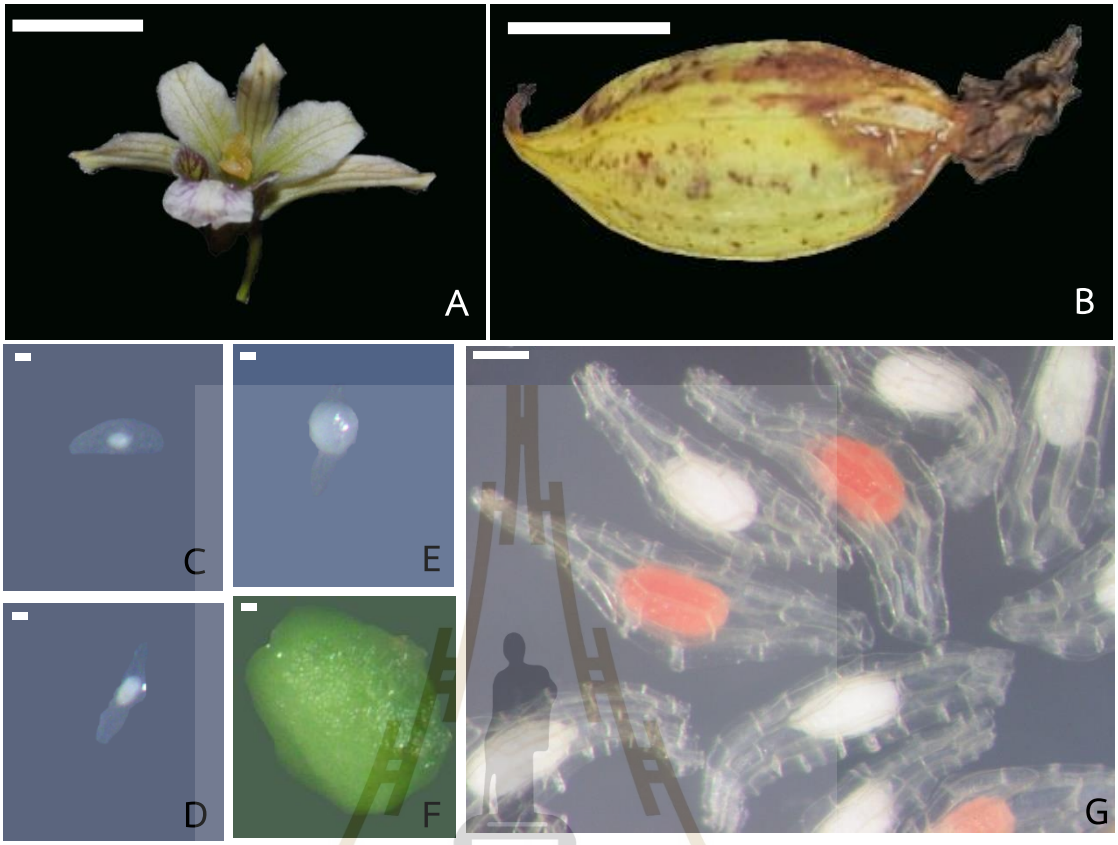

Figure 4.1 Flower, capsule, seed development and TTC treated seeds of *E.bicallosa*: A; flower, B; capsule C; Stage 0, D; Stage 1, E; Stage 2, F; Stage 3, G; treated seeds with 1% TTC (red= viability). Scale bar: (A-B) = 1 cm, (C-G) = 100 µm.

Table 4.1 Effect of Na(OCl) concentration and duration on seed viability germination, and contamination.

Concentration (%) x Time (min)	Viability (%)	Germination (%)	Contamination
0 x 10	66.90^{b}	nd	++++
0.5 x 1	91.95^{a}	91.70^{a}	+
0.5 x 5	62.06^{b}	58.89^{b}	-
0.5 x10	59.10^{b}	58.42^{b}	-
1 x 1	87.37^{a}	83.5^{a}	+
1 x 5	13.82^{c}	21.1^{c}	+
1 x 10	4.70^{c}	1.49^{c}	-

Remark: nd= not determined; Different letters indicate significant differences among treatments according to the LSD test ($p < 0.05$); “+” = contamination level; “-” = no contamination detected

4.2 Effect of culture media on seed germination and protocorm development of *E. bicallosa*

After 3 months of observation, three seed development stages were found (Figure 4.1), including Stage 1; seed swelling due to water imbibition and cell division, Stage 2; embryo getting bigger and rupture of testa due to cell division, and Stage 3; embryo greening enlargement due to chlorophyll synthesis and cell division. According to the results, seed germination and protocorm development were affected by the types and concentrations of culture media. Even though all media can support seed germination and protocorm development to Stage 3, the germination percentage and proportion of each protocorm development stage varied among media types and concentrations as in Table 4.2. From Table 4.2, the result shows that MS (92.88%) and ½MS (89.21%) gave significantly highest average seed germination followed by ½ MM, MM, ½ VW, and VW, with 80.13%, 70.67%, 80.13%, 65.51%, and 56.12%, respectively. Moreover, MS (91.41%) and ½MS (89.21%) also gave significantly highest stage 3 development percentages followed by ½ MM ½ VW, MM, and VW, with 77.91%, 65.51%, 60.88%, and 49.70% respectively. I also found that protocorms on MS (Figure 4.2E) were bigger than other media, and those on MM (Figure 4.2D) had well-developed rhizoids compared to other media.

Table 4.2 Seed germination stages of *E. bicallosa* on various media for 3 months.

Medium	Stage 0 (%)	Stage 1(%)	Stage 2 (%)	Stage 3 (%)	Total germination (%)
MM	29.32±9.3[a]	0.57±0.52[a]	9.22±1.00[a]	60.88±10.08[b]	70.67±9.38[b]
½ MM*	19.88±0.10	0.00	2.21±1.10	77.91±0.10	80.13±1.00
MS	8.90±6.68[b]	0.00[b]	1.52±0.43[b]	91.40±8.60[a]	92.88±7.02[a]
½ MS	10.79±2.41[b]	0.00[b]	0.00[c]	89.21±2.41[a]	89.21±2.41[a]
VW	43.88±9.68[a]	0.82±0.42[a]	3.63±3.31[ab]	49.70±4.81[b]	56.12±9.68[b]
½ VW	34.49±4.24[a]	2.65±2.18[a]	3.20±2.97[ab]	65.51±4.24[b]	65.51±4.24[b]

Remark: Different letters indicate significant differences in each media tested according to the LSD test ($p < 0.05$); *½ MM had only two replicate plates, unable to perform statistical analysis on this treatment

Table 4.3 Macro and micronutrients which might have a high impact on orchid seed germination and development.

Nutrients	MS	MM	VW
NH_4	**20.6 mM**	-	7.6 mM
NO_3	**39.4 mM**	-	5.2 mM
PO_4	1.2 mM	1.0 mM	3.1 mM
K	**20.0 mM**	0.6 mM	7.0 mM
Mg	**1.5 mM**	0.8 mM	1.0 mM
Glycine	0.002 g	**0.002 g**	-
Casein hydrolysate	-	**0.4 g**	-
Pineapple powder	-	**20 g**	-
Activated charcoal	-	**1 g**	-
Sucrose	**30 g**	10 g	20 g

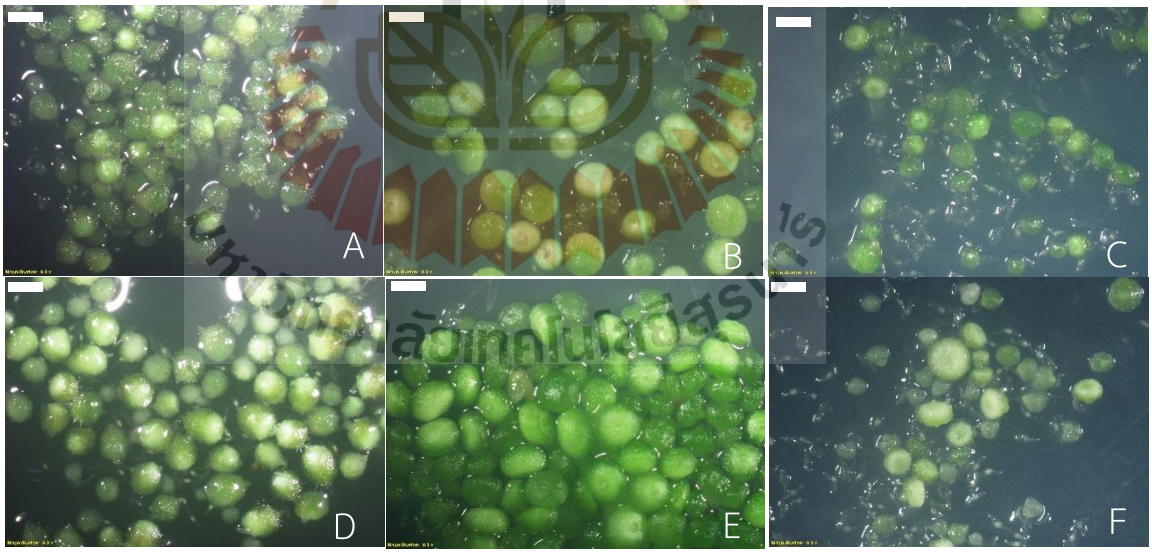

Figure 4.2 Seed germination on various media at 3 months after sowing: A; 1/2MM, B; 1/2MS, C; 1/2VW, D; MM, E; MS, F; VW (scale bar= 1 mm).

The reason why MS and ½MS gave significantly the highest average seed germination and stage 3 seed development percentage is that MS contains more macro-micronutrients than MM and VW. Notably, MS contains higher NH_4 and NO_3, which are crucial for orchid seed germination (Fang et al., 2019). Additionally, Quiroz et al. (2017) reported that high levels of K and Mg are essential for chlorophyll synthesis and stage 3 seed development involving greening enlargement due to chlorophyll synthesis and cell division. Furthermore, the presence of higher sucrose in MS serves as a carbon and energy source for cell division (Table 4.3). However, according to Table 4.2 and Table 4.3, MM, and ½MM gave higher average seed germination and stage 3 seed development percentages than VW and ½VW, despite lacking NH_4, NO_3, and having lower levels of PO_4, K, Mg, and sucrose. This may be attributed to the presence of glycine and casein hydrolysate in MM, as these two compounds can serve as organic nitrogen sources, potentially replacing the need for NH_4 and NO_3. Additionally, pineapple powder in the media can supply various trace elements such as PO_4, K, Mg, and other nutrients such as sucrose and organic nitrogen. The inclusion of activated charcoal in the media may also play a role in stimulating root and rhizoid development while increasing nutrient absorption.

4.3 Effect of colchicine concentration and duration time on polyploid induction of *E. bicallosa*

Hyperhydricity accidentally occurred in 0 (w/v) colchicine treatment (control treatment), due to hypotonicity of DI water (Ueno, Cheplick and Shetty, 2001) or oxidative stress from subculturing, resulting in reduced survival rate by tissue necrosis (Cassel and Curry, 2001) from 100% to 95.56%, 86.11%, and 77.22%, from first month to third month respectively (Table 4.3), (Figure 4.3). Hyperhydricity also affected plant development in the control treatment, even though all survived protocorm in could develop to rhizome-like bodies stage like other treatments, however, plants in this treatment could not develop shoot or root, which may be caused by reduced plant regenerated capability (Jan et al., 2023), ruined apical dominance and callusing from hyperhydricity (Cassel and Curry, 2001). 0.1% colchicine treatment gave a slight decrease in survival rate to 88.33%

compared to 0.025% and 0.05% colchicine treatments which gave a 100% survival rate (Table 4.3), (Figure 4.3), which may be caused by high concentration colchicine toxicity, which can lead to tissue browning (Chung et al., 2017), decreased plant survival (Gantait, Mandal, Bhattacharyya and Das, 2011), and high mortality rate (Eng and Ho, 2019). The plant development is different among colchicine treatments (0.025%, 0.05%, and 0.1%), even though all survived protocorms could develop to rhizome-like bodies stage (Table 4.4), (Figure 4.3), however, shoot and root development varied among treatments (Figure 4.4), and trended to be inhibited in 0.1% colchicine treatment (giving the lowest number on most parameters, except average root induction on the third month). On the other hand, the shoot and root development were enhanced in 0.025% and 0.05% colchicine treatments. In the second month, plants in 0.025% colchicine treatment had better shoot regeneration, yielding the highest average shoot per plant (1.28 shoots), shoot induction rate (18.33%), root per plant (1.59 roots), and root induction rate (16.67%) than 0.05% colchicine treatment, while 0.05% colchicine treatment had the highest average length of the longest shoot (1.91 cm), and root (0.80 cm). However, in the third month, 0.05 (w/v) colchicine treatment could catch up with 0.025% colchicine treatment and even turned into the best treatment for shoots and roots development, giving the highest number on most parameters, except average root per plant (Table 4.5, Table 4.6). For the effect of colchicine treatment on leaf anatomy, plants treated with 0.025% colchicine had a significantly lower average number of leaf epidermal cells per area (43.78 cells per 0.1 mm^2) than plants in the control treatment, plants treated with 0.05% colchicine had significantly longer average stomatal length (36.32 μm) and width (30.41 μm) than plants in the control treatment. There is no significant difference between the number of stomata per area from each treatment, and plants treated with 0.1% colchicine had significantly longer average stomatal width (30.81 μm) than plants in the control treatment (Figure 4.4, Table 4.7, 4.8). The variety of plant development, morphology, and anatomy among colchicine treatments might come from genome duplication that caused an enormous change in gene expression (increasing/silencing, up/downregulation) (Niazian and Nalousi, 2020). Epigenetic (chromosome rearrangements, alteration of regulators network) and loss of duplicated genes might lead to unexpected, unpredictable, and

nonlinearity results such as increase or decrease plant high, faster or slower growth rate, bigger or smaller flower (Lannicelli et al., 2020), increase or decrease (Hong, Hwang and Lim, 2020; Ari, Djapo, Mutlu, Gurbuz and Karaguzel, 2015), leaf length and width, early) or delay in flowering (Zahumenicka et al. 2017; Mo et al., 2020) and change in leave shape (Hoang et al., 2020).

Table 4.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment.

Month (After colchicine treatment)	Colchicine treatment % (w/v)	Survival rate (%)
1	0.00	95.56 ± 4.55^{ab}
1	0.025	100.00 ± 0.00^{a}
1	0.05	100.00 ± 0.00^{a}
1	0.10	88.33 ± 9.83^{bc}
2	0.00	86.11 ± 4.91^{c}
2	0.025	100.00 ± 0.00^{a}
2	0.05	100.00 ± 0.00^{a}
2	0.10	88.33 ± 4.71^{bc}
3	0.00	77.22 ± 14.67^{c}
3	0.025	100.00 ± 0.00^{a}
3	0.05	100.00 ± 0.00^{a}
3	0.10	88.33 ± 4.59^{bc}

Remark: Different letters indicate significant differences in each colchicine treatment in the same column, according to the LSD test ($p < 0.05$)

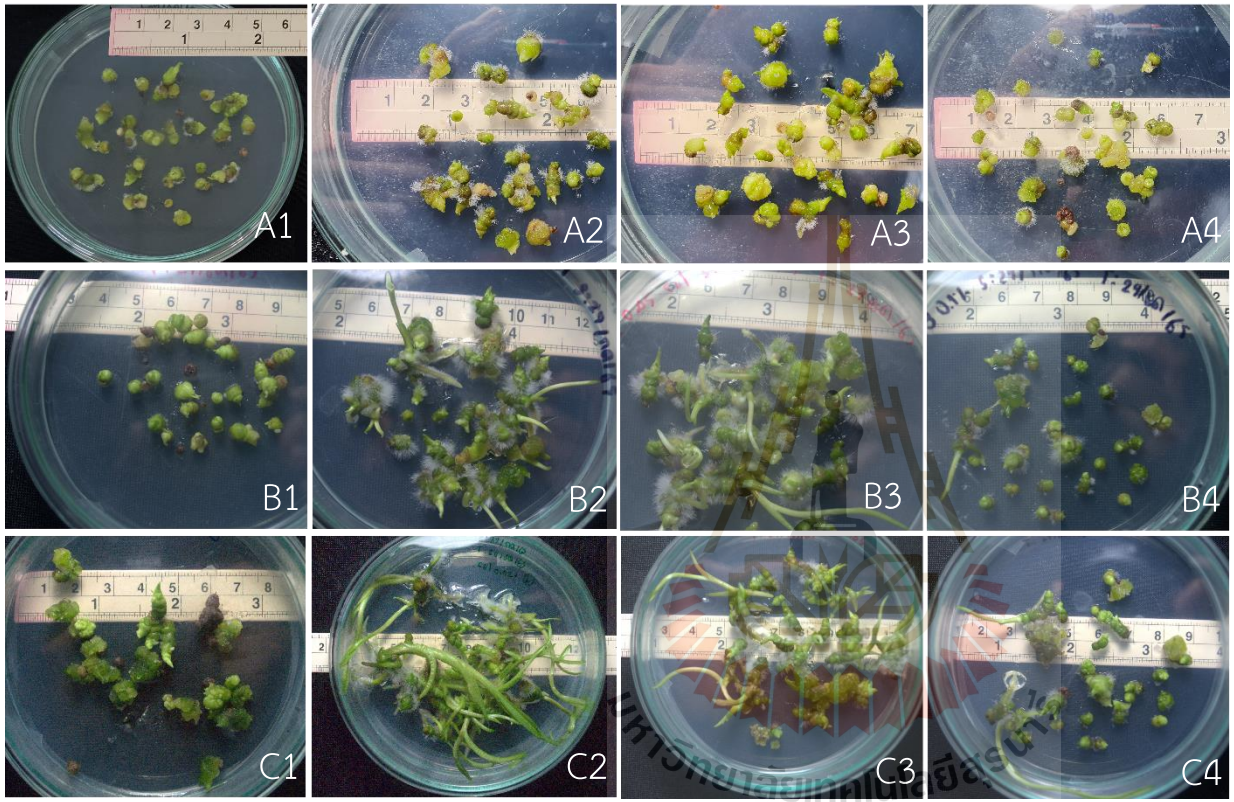

Figure 4.3 Protocorm development on various colchicine treatments for 3 months: plant after treated with 0%, 0.025%, 0.05%, and 0.1% colchicine respectively on the first month (A1-A4), the second month (B1-B4), and the third month (C1-C4).

Table 4.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa*.

Month (After colchicine treatment)	Colchicine treatment % (w/v)	Stage 3 (%)	Stage 4 (%)	Stage 5 (%)	Rhizome-like body (%)
1	0	95.56 ± 4.55^{a}	0.00^{b}	0.00^{b}	0.00^{d}
1	0.025	79.17 ± 11.01^{b}	17.50 ± 9.95^{a}	3.33 ± 2.72^{a}	0.00^{d}
1	0.05	82.78 ± 12.90^{b}	15.00 ± 12.25^{a}	0.00^{b}	0.00^{d}
1	0.10	88.33 ± 9.83^{ab}	0.00^{b}	0.00^{b}	0.00^{d}
2	0	0.00^{c}	0.00^{b}	0.00^{b}	86.11 ± 4.91^{b}
2	0.025	0.00^{c}	0.00^{b}	0.00^{b}	100.00 ± 0.00^{a}
2	0.05	0.00^{c}	0.00^{b}	0.00^{b}	100.00 ± 0.00^{a}
2	0.10	0.00^{c}	0.00^{b}	0.00^{b}	88.33 ± 4.71^{b}
3	0	0.00^{c}	0.00^{b}	0.00^{b}	77.22 ± 14.67^{c}
3	0.025	0.00^{c}	0.00^{b}	0.00^{b}	100.00 ± 0.00^{a}
3	0.05	0.00^{c}	0.00^{b}	0.00^{b}	100.00 ± 0.00^{a}
3	0.1	0.00^{c}	0.00^{b}	0.00^{b}	88.33 ± 4.59^{b}

Remark: Different letters indicate significant differences in each colchicine treatment in the same column, using The ANOVA with LSD for post hoc test ($p<0.05$)

Table 4.6 Influence of colchicine treatment on shoot development of *E. bicallosa*.

Month (After colchicine treatment)	Colchicine treatment % (w/v)	Shoots per plant (no. of shoot)	Length of the longest shoot (cm)	Shoot induction rate (%)
1	0.00	0.00^{c}	0.00^{e}	0.00^{e}
1	0.025	1.00^{b}	0.19 ± 0.15^{de}	14.00 ± 6.83^{cd}
1	0.05	1.00^{b}	0.28 ± 0.23^{de}	7.33 ± 2.80^{de}
1	0.10	0.00^{c}	0.00^{e}	0.00^{e}
2	0.00	0.00^{c}	0.00^{e}	0.00^{e}
2	0.025	1.28 ± 0.20^{a}	1.51 ± 0.78^{bc}	18.33 ± 8.10^{cd}
2	0.05	1.16 ± 0.20^{ab}	1.91 ± 1.1^{bc}	13.33 ± 10.10^{cd}
2	0.10	1.00^{b}	1.04 ± 0.48^{cd}	10.00 ± 8.83^{de}
3	0.00	0.00^{c}	0.00^{e}	0.00^{e}
3	0.025	1.23 ± 0.17^{a}	3.98 ± 1.09^{a}	30.00 ± 12.47^{ab}
3	0.05	1.24 ± 0.21^{a}	4.15 ± 1.04^{a}	35.33 ± 17.60^{a}
3	0.10	1.22 ± 0.21^{a}	2.30 ± 1.31^{b}	21.67 ± 5.77^{bc}

Remark: Different letters indicate significant differences in each colchicine treatment in the same column, using an ANOVA with LSD for post hoc test ($p<0.05$)

Table 4.7 Root development of plant treated with various colchicine concentrations.

Month (After colchicine treatment)	Colchicine treatment % (w/v)	Roots per plant (root)	Length of the longest root (cm)	Root induction rate (%)
1	0.00	0.00^{d}	0.00^{d}	0.00^{d}
1	0.025	0.00^{d}	0.00^{d}	0.00^{d}
1	0.05	0.00^{d}	0.00^{d}	0.00^{d}
1	0.10	0.00^{d}	0.00^{d}	0.00^{d}
2	0.00	0.00^{d}	0.00^{d}	0.00^{d}
2	0.025	1.59 ± 0.32^{ab}	0.75 ± 0.46^{bcd}	16.67 ± 6.23^{b}
2	0.05	1.40 ± 0.34^{ab}	0.80 ± 0.31^{bc}	13.90 ± 5.73^{bc}
2	0.10	1.00^{c}	0.30 ± 0.27^{cd}	9.17 ± 4.20^{c}
3	0.00	0.00^{d}	0.00^{d}	0.00^{d}
3	0.025	1.61 ± 0.26^{a}	1.18 ± 0.16^{b}	13.33 ± 4.07^{bc}
3	0.05	1.49 ± 0.35^{ab}	2.51 ± 1.84^{a}	21.67 ± 4.30^{a}
3	0.10	1.35 ± 0.20^{b}	0.90 ± 0.27^{bc}	15.57 ± 8.60^{b}

Remark: Different letters indicate significant differences in each colchicine treatment in the same column, using an ANOVA with LSD for the post hoc test ($p<0.05$)

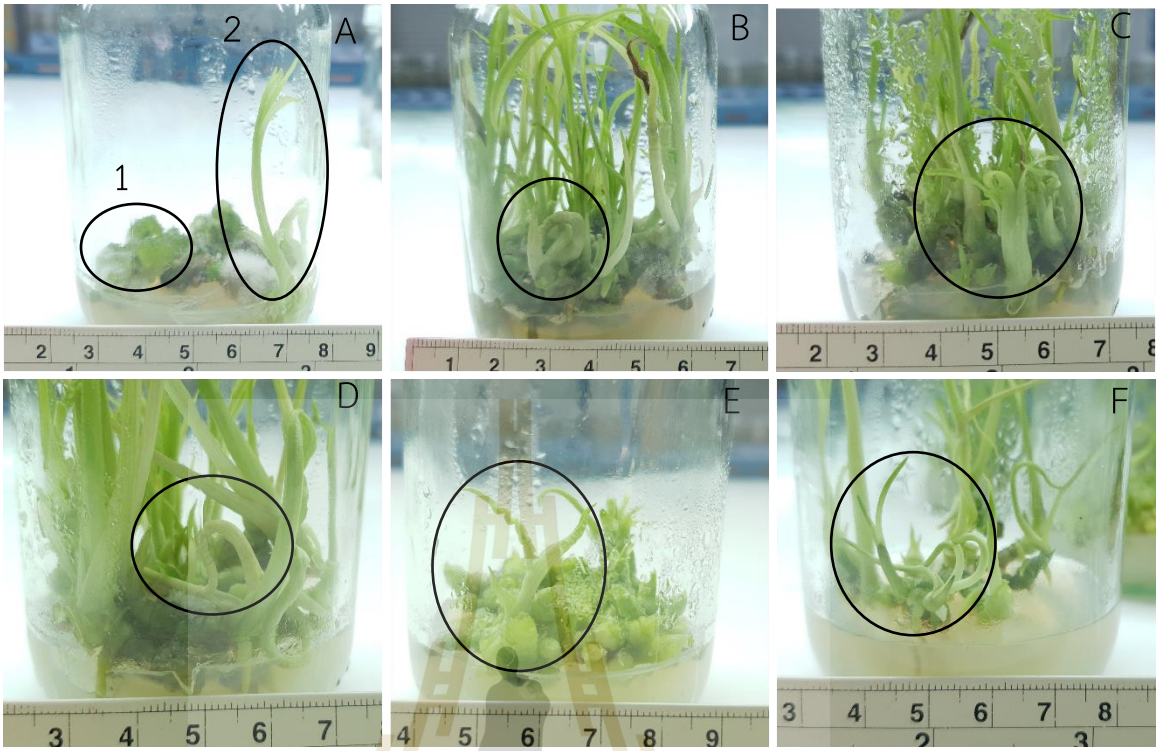

Figure 4.4 Plant development on various colchicine concentrations: A1; abnormal development plant in control treatment due to hyperhydricity, A2; normal development plant in control treatment, B-C; suspected polyploid plant with abnormal development from 0.025% colchicine treatment, D; suspected polyploid plant with abnormal development from 0.05% colchicine treatment, E-F; suspected polyploid plant with abnormal development from 0.1% colchicine treatment.

Table 4.8 The number of leaf epidermal cells and stomata of colchicine-treated plants.

Colchicine treatment % (w/v)	Number of leaf epidermal cells per 0.1 mm^2	Number of stomata per 0.1 mm^2
0.00	55.22 ± 11.45^{ab}	5.81 ± 2.02^{a}
0.025	43.78 ± 10.24^{c}	4.74 ± 1.81^{a}
0.05	51.93 ± 10.58^{b}	5.04 ± 1.16^{a}
0.10	59.44 ± 13.53^{a}	6.00 ± 2.24^{a}

Remark: Different letters indicate significant differences in each colchicine treatment in the same column, using an ANOVA with DMRT for the poshoc test ($p<0.05$) to comparing between number of leaf epidermal cells and Kruskal-Wallis to comparing between number of stomata.

Table 4.9 The stomatal length and width of colchicine-treated plants.

Colchicine treatment % (w/v)	Stomatal length (um)	Stomatal width (um)
0.00	32.84 ± 2.91^{c}	28.24 ± 2.57^{b}
0.025	35.79 ± 4.81^{ab}	30.27 ± 4.41^{a}
0.05	36.32 ± 4.85^{a}	30.41 ± 3.86^{a}
0.10	34.13 ± 2.64^{bc}	30.81 ± 3.28^{a}

Remark: Different letters indicate significant differences in each colchicine treatment in the same column, using a Kruskal-Wallis test ($p<0.05$).

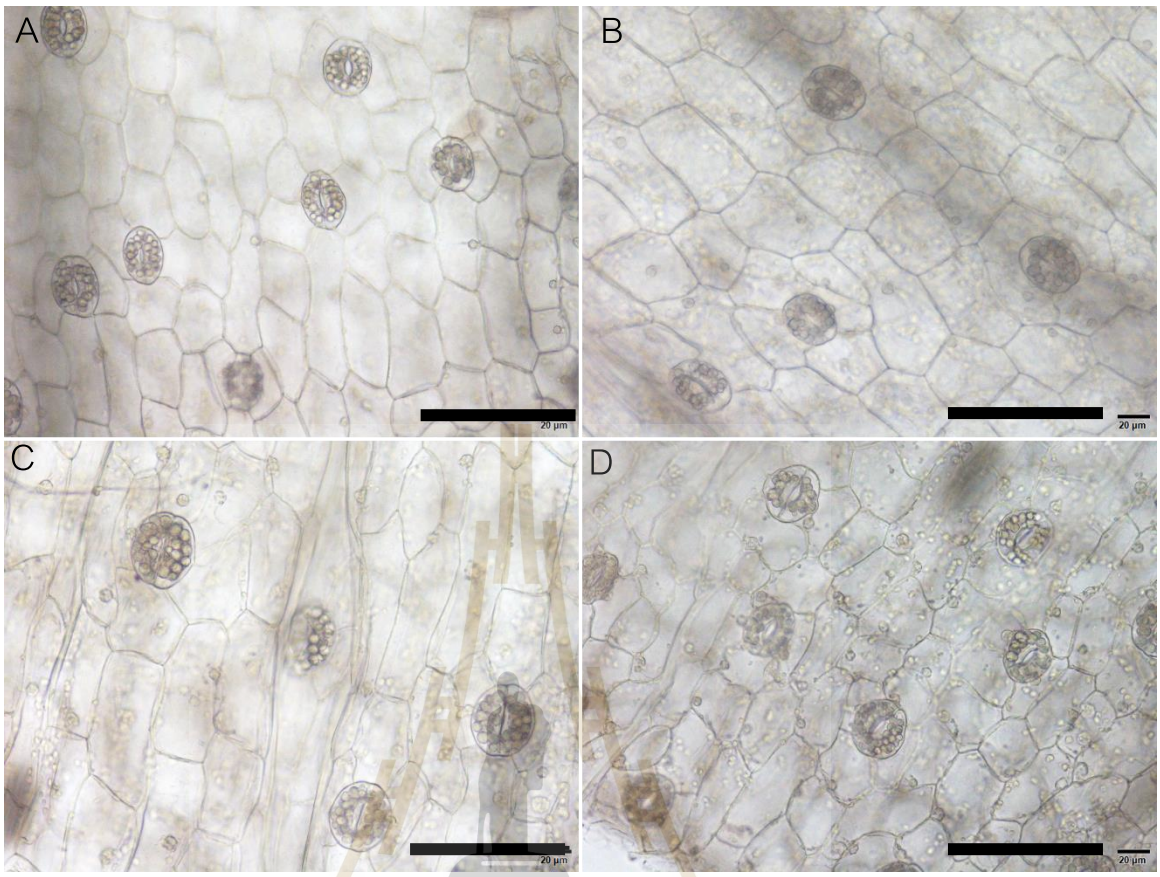

Figure 4.5 Leaf epidermis of colchicine treated plant: A; control plant, B; 0.025% colchicine treated plant, C; 0.05% colchicine treated plant, D; 0.1% colchicine treated plant (Scale bar = 100 µm.).

The effect of colchicine treatment on plant cell DNA contents was investigated in this study. The DNA content of seedlings was analyzed using a Quantum Analysis Flow cytometer (model: QA FCM QP 2, USA) by setting a 2C DNA content peak at channel 150. The flow cytometry results (Figure 4.6) were analyzed following the method described by Zonneveld and Pollock (2012). The analysis showed that the control plant contains cells with 3 different DNA contents including 2C at channel 150, 2.6C at channel 200, and 4C at channel 300 (Figure 4.4A). From this result, this species could be classified as a euploid-aneuploid mosaic organism, containing both euploid (2C and 4C) and aneuploid (2.6C) cells (Danial and Pradi, 2023). This phenomenon can occur naturally (Gola, Zolubak, Lobas and Jakubska-Busse, 2019), (Sharma and Mukai, 2015), (Zonneveld and Pollock, 2012) or be induced through polyploidization (Frank

and Chitwood, 2016). Colchicine treatment at high concentrations (0.05% and 0.1%) has the potential to create cells with 5.3C DNA content, as observed at channel 400 (Figure 4.6C and 4.6D). These cells may arise from the chromosome duplication of cells with 2.6C DNA content due to the effect of colchicine. This finding corresponds to previous reports indicating that colchicine can induce the formation of polyploid cells by interrupting chromosome separation during anaphase (Eng and Ho, 2019). Moreover, many researchers have reported the creation of new cytotypes with increased DNA content in the tissue through colchicine treatments in various species, including *Dendrobium chrysotoxum* (4n) (Atichart, 2013), *Phalaenopsis amabilis* (L.) Blume (4n) (Azmi et al., 2016), *Paphiopedilum villosum* (4n) (Huy et al., 2019), *Dendrobium cariniferum* (16c) (Zhang and Gao, 2020).

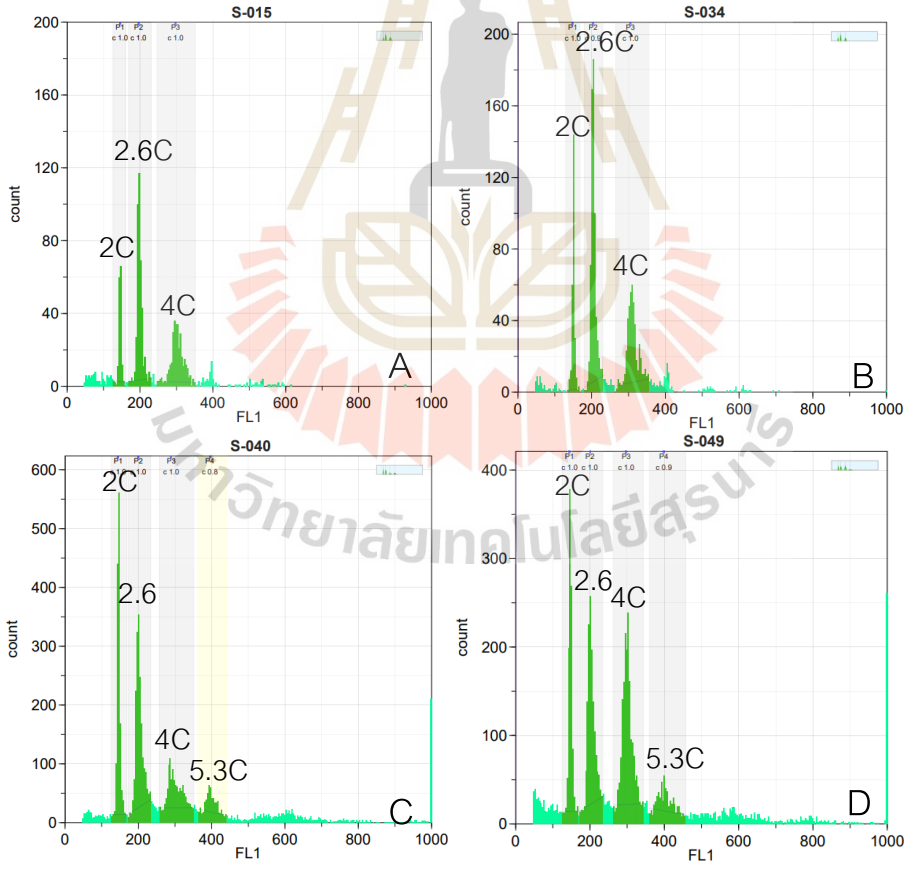

Figure 4.6 Effect of colchicine treatments on DNA content of plant cells: A-D, a population of plant cells from seedlings delivered 0 (control), 0.025%, 0.05%, and 0.1% treated protocorm respectively. 2c peak was set at channel 150.

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 The effect of Na(OCl) concentration and treatment duration on seed viability and germination of *E. bicallosa*

To eliminate all microbes from the seeds, a sufficient Na(OCl) concentration and treatment duration are needed. However, it is essential to be cautious as Na(OCl) not only eliminates microbes but also causes damage to the seed tissue (Yildiz, Ozcan, Kahramanogullari, and Tuna, 2012). Treating with excessively high concentrations or for prolonged durations can lead to excessive damage to the seed tissue, ultimately inhibiting germination. For this study, I chose a 0.5% Na(OCl) concentration treated for either 5 or 10 min. These conditions resulted in an acceptable level of tissue damage (59-62% average viability and 58% germination) with good contamination control (no contamination detected).

5.2 Effect of culture media on seed germination and protocorm development of *E. bicallosa*

Media types and concentrations are also crucial for orchid seed germination and development. Using improper media may lead to abnormal development (Decruse et al., 2013), growth stunting, failure to develop into a seedling (Nanekar et al., 2014), and failure to germinate (McAlister and Staden, 1998). In this study, all media supported seed germination and development to the same stage. However, there were variations in the morphology of protocorms among media types. Protocorms grown on MS exhibited bigger sizes than those on other media, while protocorms on MM had well-developed rhizoids compared to other media. Considering the results, MS and ½MS were chosen as the optimal media since they resulted in the highest average seed germination and stage 3 development percentage.

5.3 Effect of colchicine concentration and duration time on polyploid induction of *E. bicallosa*

The hyperhydricity accidentally occurred in the control treatment due to the hypotonicity of DI water (Ueno, Cheplick and Shetty, 2001) or oxidative stress from subculturing, resulting in a reduction in survival rate by tissue necrosis (Cassel and Curry, 2001) (100%-77.22% in the third month). This condition inhibited shoot and root development as the plant's regenerated capability decreased (Jan et al., 2023), and it also ruined apical dominance and caused callusing (Jan et al., 2023). The survival rate and development of the plants varied among the colchicine treatments (0.025%, 0.05%, and 0.1%). Higher colchicine concentrations might cause colchicine toxicity, which leads to tissue browning (Chung et al., 2017), decreased plant survival (Gantait, Mandal, Bhattacharyya and Das, 2011), and a high mortality rate (Eng and Ho, 2019). For instance, in the 0.1% colchicine treatment, the survival rate decreased from 100% to 88.33%. Polyploidization of colchicine also caused an enormous change in gene expression (increasing/silencing, up/downregulation) (Niazian and Nalousi, 2020), epigenetic (chromosome rearrangements, alteration of regulators network), and loss of duplicated genes. These changes might lead to unexpected, unpredictable, and nonlinearity results such as an increase or decrease in plant high, faster or slower growth rate, bigger or smaller flower (Iannicelli et al., 2020), as well as variations in leaf length and width-either increased (Hoang, Hwang and Lim, 2020) or decreased (Ari, Djapo, Mutlu, Gurbuz and Karaguzel, 2015). Additionally, polyploidization might lead to early (Zahumenická et al., 2017) or delayed (Mo et al., 2020) flowering and changes in leaf shape (Hoang et al., 2020). The findings of this study align with previous reports when comparing shoot, root development, and leaf anatomy. Under 0.025%, 0.05%, and 0.1% colchicine treatments. In the case of 0.1% colchicine treatment, shoot, root development trended to be inhibited, with shoot and root starting to develop shoot and on the second month and giving the lowest numbers for most parameters, except for average root induction on the third month. In the case of 0.1%, colchicine treatment seems to have a little effect on leaf anatomy by giving a significantly longer

stomatal width than the control treatment. On the other hand, enhancing shoot, root development, and changes in leaf anatomy are found in 0.025%, 0.05% colchicine treatments, in these cases shoot and root development starting to develop in the first month and the 0.05% colchicine treatment proved to be the best effective for both shoots and roots development, giving the highest numbers for most parameters, except for average root per plant, colchicine treatment of 0.025% seem to had bigger leaf epidermal cell than control treatment (significantly lower number of leaf epidermal cells per area than control treatment) and 0.05% colchicine treatment had bigger stomatal size than control treatment (significantly higher stomatal length and width than control treatment).

E. bicallosa species is a euploid-aneuploid mosaic organism, due to the possession of euploid (2C and 4C) and aneuploid (2.6C) cells within the tissues (Danial and Pradi, 2023). Colchicine treatments at high concentrations (0.05% and 0.1%) can create a new type of cell with higher DNA content, 5.3C (detected at channel 400), which might arise from the chromosome duplication of 2.6C DNA content cells by the effect of colchicine that interrupts chromosome separation during anaphase (Eng and Ho, 2019). These results also correspond to previous reports which have reported the creation of new cell types with increased DNA content following colchicine treatments, as observed in chrysotoxum (4n) (Atichart, 2013), *Phalaenopsis amabilis* (L.) Blume (4n) (Azmi et al., 2016), *Paphiopedilum villosum* (4n) (Huy et al., 2019), and *Dendrobium cariniferum* (16c) (Zhang and Gao, 2020).

Future directions

In this study, the fully developed embryos (seedlings) and the long-term effect of colchicine on embryo and seedling development have not yet been obtained. However, the effect of Na(OCl) on seed viability and germination, the influence of culture media on seed germination and early development, and the effect of colchicine on DNA content were already investigated. Future work should focus on exploring the effects of culture media on late embryo and seedling development, modifying media composition to get a better result, and investigating the long-term effect of colchicine on later embryo development stage, seedling development, and morphology.

REFERENCES

REFERENCES

Ari, E., Djapo, H., Mutlu, N., Gurbuz, E. and Karaguzel, O. (2015). Creation of variation through gamma irradiation and polyploidization in *Vitex agnus-castus* L. *Scientia Horticulturae*, *195*, 74–81.

Atichart, P. (2013). Polyploid induction by colchicine treatments and plant regeneration of *Dendrobium chrysotoxum*. *Thai Journal of Agricultural Science*, *46*(1), 59-63.

Azmi, K., Sukma, D., Aziz, A. and Syukur. M. (2016). Polyploidy induction of moth orchid (*Phalaenopsis amabilis* (L.) Blume) by colchicine treatment on pollinated flowers. *The Journal of Agricultural Sciences*, *11*, 62-73.

Baro, D., Bawri, A., Adhikari, A. and Borthakur, S.K. (2019). Orchid flora of Manas National Park, India. *Asian Journal of Conservation Biology*, *8*(2), 143-148.

Cassel A.C. and Curry R.F. (2001). Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell, Tissue and Organ Culture*, *64*, 144-157.

Chen, W.H. and Tang, C.Y. (2018). A protocol for the induction of polyploids in *Phalaenopsis* orchids by in vitro method without using anti-microtubule agents. orchid propagation: From laboratories to greenhouses -methods and protocols, 317-330.

Chen, X., Cribb, P.J. and Gale S.W. (2009). *Eulophia*. Flora of China, 25, 253.

Chen, X., Liu, Z., Zhu, G., Lang, K., Ji, Z., Luo, Y., Jin X., Cribb, J., Wood, J., Gale, S., Ormerod, P., Vermeulen, J., Wood, H., Clayton, D. and Bell, A. (2009). *Eulophia bicallosa*. Flora of China, *25*, 254.

Choopeng, S., Te-chato, S. and Khawnium, T. (2019). Effect of colchicine on survival rate and ploidy level of hybrid between *Dendrobium santana* x *D. friedericksianum* orchid. International *Journal of Agricultural Technology*, *15*(2), 249-260.

Chung, H., Shi, S., Huang, B. and Chen, J. (2017). Enhanced agronomic traits and medicinal constituents of autotetraploids in *Anoectochilus formosanus* Hayata, *a top-grade medicinal orchid. Molecules*, *22*, 1-13.

Danial, A.Q.M. and Pradip, R.C. (2023). StatPearls. United state: StatPearls Publishing.

Darzynkiewicz, Z. and Juan, G. (1997). DNA content measurement for DNA ploidy and cell cycle analysis. *Current Protocols in Cytometry*, *7*, 1-24.

Decruse, W., Reny, N., Shylajakumari, S. and Krishnan, N. (2013). In vitro propagation and field establishment of *Eulophia cullenii* (Wight) Bl., a critically endangered orchid of Western Ghats, India through culture of seeds and axenic seedling-derived rhizomes. *In Vitro Cellular & Developmental Biology – Plant*, *49*, 520-528.

Department of Environment and Science, Queensland (2013) Rare or threatened plants of Hinchinbrook Island National Park, WetlandInfo website, accessed 30 October 2023. Available at: https://wetlandinfo.des.qld.gov.au/wetlands/facts-maps/wildlife/?AreaID=national-park-hinchinbrook-island&Kingdom island&Kingdom=plants&SpeciesFilter=RareOrThreatened

Eng, W. and Ho, W. (2019). Polyploidization using colchicine in horticultural plants: A review. *Scientia Horticulturae*, *246*, 604-617.

Fang, J. and Berrientos B.A.B. (2019). Influence of photoperiod and culture medium on the speed of asymbiotic seed germination and seedling development in *Spathoglottis plicata. HortScience*, *54*(9), 1570-1575.

Frank, M.H. and Chitwood, D.H. (2016). Plant chimeras: The good, the bad, and the "Bizzaria." *Developmental Biology*, *419*(1), 41–53.

Gantait, S., Mandal, N., Bhattacharyya, S. and Das, P.K. (2011). Induction and identification of tetraploids using in vitro colchicine treatment of *Gerbera jamesonii* Bolus cv. *Sciella. Plant Cell, Tissue and Organ Culture*, *106*(3), 485-493.

Gola, E.M., Zolubak, E., Lobas, L. and Jakubska-Busse, A. (2019). Taxonomic treatment of chimeras in Orchids. *Annales Botanici Fennici*, *1*(56), 395-402.

Hoang, T. K., Hwang, Y.-J., and Lim, J.-H. (2020). Chemical polyploidization of *Chrysanthemum boreale*. *Plant Cell, Tissue and Organ Culture*, *140*(3), 677–683.

Huy, N.P., Tam, D.T.T., Luan, V.Q., Tung, H.T., Hien, V.T., Ngan, H.T.M., Duy, P.N. and Nhut, D.T. (2019). In vitro polyploid induction of *Paphiopedilum villosum* using colchicine. *Scientia Horticulturae*, *252*, 283-290.

Jan, T., Gul S., Khan A., Noor A., Amin H., Bibi S., Khan A.R., Ahmad M.S., Azm R. and Ullah H. (2023). Range of factors in the reduction of hyperhydricity associated with in vitro shoots of *Salvia santolinifolia* Bioss. *Brazilian Journal of Biology*, (83), 1-8.

Johnson, T., Stewart, S., Dutra, D., Kane, M. and Richardson, M. (2007). Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae) - Preliminary evidence for the symbiotic culture advantage. *Plant Cell, Tissue and Organ Culture*, *90*, 313-323.

Li, C., Dong, N., Zhao, Y., Wu, S., Liu, Z. and Zhai, J. (2021). A review for the breeding of orchids: Current achievements and prospects. *Horticultural Plant Journal*, *7*(5), 380-392.

Kauth, P.J., Dutra, D., Johnson, T.R., Stewart, S.L., Kane, M.E. and Vendrame, W. (2014). Techniques and applications of in vitro orchid seed germination. *Floriculture, Ornamental and Plant Biotechnology*, *5*, 375-391.

Iannicelli, J., Guariniello, J., Tossi, V.E., Regalado, J.J., Di Ciaccio, L., Van Baren, C.M., Pitta Alvarez, S.I. and Escandon, A.S. (2020). The “polyploid effect” in the breeding of aromatic and medicinal species. *Scientia Horticulturae*, *260*, 1-10.

Mo, L., Chen, J., Chen, F., Xu, Q., Tong, Z., Huang, H. and Lin, E. (2020). Induction and characterization of polyploids from seeds of *Rhododendron fortunei* Lindl. *Journal of Integrative Agriculture*, *19*(8), 2016–2026.

Nanekar, V., Shriram, V., Kumar, V. and Kavi Kish, B. (2014). Asymbiotic in vitro seed germination and seedling development of *Eulophia nuda* Lindl., an endangered medicinal orchid. *Proceedings of the National Academy of Sciences*, India - Section B: Biological Sciences, (84), 837-846.

Narkhede, A., Mahajan, M., Singh, E., Harsulkar, A. and Jagtap, S. (2016). Antioxidant activity of fourteen *Eulophia* species traditionally known as amarkand. *International Journal of Pharmacy and Pharmaceutical Sciences*, *8*, 313-316.

Niazian, M. and Nalousi, M. (2020). Artificial polyploidy induction for improvement of ornamental and medicinal plants. *Plant Cell, Tissue and Organ Culture*, *142*, 447-469.

Malmgren, S. (1996). Orchid propagation: theory and practice. In Allen C (ed.), North American native Terrestrial orchids: propagation and production. *Symposium conducted at the meeting of North American Native Terrestrial Orchid Conference*, Germantown, Maryland.

McAlister, G. and Staden, V. (1998). In vitro culture of *Eulophia* species. *South African Journal of Botany*, (64), 264-266.

Murashige, T. and Nakano, R. (1966). Tissue culture as a potential tool in obtaining polyploid plants. *Journal of Heredity*, *57*, 114-118

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarump*, *15*(3), 473-497.

Quiroz, K., Saavedra, J., Vogel, H., Verdugo, G., Caligari, P.D.S. and Gonzales, R.G. (2017). In vitro asymbiotic germination for micropropagation of the recalcitrant terrestrial orchid *Chloraea crispa. Application in Plant Science*, *5*(8), 1-9.

Sattler, C., Carvalho, R. and Clarindo, R. (2015). The polyploidy and its key role in plant breeding. *Planta*, *2*, 281-296.

Sharma, S.K. and Mukai, Y. (2015). Chromosome research in orchids: current status and future prospects with special emphasis from molecular and epigenetic perspective. *The Nucleus*, *58*(3), 173–184.

Stewart, L. and Zettler, W. (2002). Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens*, *H. quinquiseta*, *H. macroceratitis*) from Florida. *Aquatic Botany*, *72*, 25-35.

Taylor, T.N., Taylor, E.L. and Krings, M. (2009). Paleobotany (Second Edition). The Biology and Evolution of Fossil Plants. *United States: Academic Press*.

Ueno K., Cheplick S. and Shetty K. (2001) Reduced hyperhydricity and enhanced growth of tissue culture-generated raspberry (Rubus sp.) clonal lines by *Pseudomonas* sp. isolated from oregano. *Process Biochemistry*, *33*, 441-445.

Vacin, F. and Wen, W. (1949). Some pH Changes in Nutrient Solutions. *Botanical Gazette*, *110*, 605-613.

Van Vuuren, R., Visagie, M., Theron, A. and Joubert, A. (2015). Antimitotic drugs in the treatment of cancer. *Cancer Chemother Pharmacol*, *76*, 1101-1112.

Yildiz, M., Ozcan, S.F., Kahramanogullari, C.T., and Tuna, E. (2012). The effect of sodium hypochlorite solutions on the viability and in vitro regeneration capacity of the tissue. *The Natural Products Journal*, *2*(4), 328–331.

Zahumenická, P., Fernández, E., Šedivá, J., Žiarovská, J., Ros-Santaella, J. L., Martínez-Fernández, D. and Milella, L. (2017). Morphological, physiological and genomic comparisons between diploids and induced tetraploids in *Anemone sylvestris* L. *Plant Cell, Tissue and Organ Culture*, *132*(2), 317–327.

Zhang, X. and Gao, J. (2020). Colchicine-induced tetraploidy in *Dendrobium cariniferum* and its effect on plantlet morphology, anatomy and genome size. *Plant Cell, Tissue and Organ Culture*, *144*, 409-420.

Zonneveld, B.J.M. and Pollock, W.I. (2012). Flow cytometric analysis of somaclonal variation in lineages of Hosta sports detects polyploidy and aneuploidy chimeras. *Plant Biology*, *14*(6), 972–979.

APPENDICES

APPENDIX A

PUBLISHED PROCEEDING

The Effect of Sodium Hypochlorite and Culture Medium on Seed Viability and Germination of *Eulophia bicallosa* (D. Don) Hunt & Summerh (Orchidaceae)

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Abstract: *Eulophia bicallosa* (D. Don) Hunt & Summerh., a terrestrial orchid having light green or cream color flowers with purplish markings, is considered a near-threatened species in Australia. In Thailand, it is recognized as a rare species since just two populations have been reported so far. The information on its propagation via seed is required. The objective of this study was to investigate seed characteristics and the effect of sterilant and culture medium on seed viability and germination of *E. bicallosa*. Seeds were sterilized in sodium hypochlorite (NaClO) at three concentrations (0, 0.5, and 1%) and three durations (1, 5, and 10 min), followed by 1%TTC for viability test. Sterilized seeds were sown on Vacin and Went (VW) media for one month for germination assessment. In addition, the effect of six medium types: MM, ½ MM, MS, ½ MS, VW, and ½ VW were assessed for seed germination for 3 months. The result showed that the seeds of *E. bicallosa* weighed only 4.50±0.29 µg per seed, with a length of 628.32 ± 9.15 µm, and a width of 195.68 ± 4.15 µm in size. At one month after sowing, seed treatments with 0.5% NaClO for 5 min or 10 min resulted in high seed viability of 62.06% and 59.10% with seed germination of 58.89% and 58.42%, respectively, without contamination. Among six media tested, the fastest and highest seed germination percentage was observed on MS and ½ MS medium in which protocorm development reached to stage 3 at 3 months after culture. Suitable sterilization and medium are required for this orchid species for optimum germination and growth.

Keywords: *asymbiotic germination, in vitro, Orchidaceae, survival rate, rare species*

1. Introduction

Orchidaceae (orchids) is a family of herbaceous plants that can be found on trees, rocks, and soil. They have a unique and beautiful flower that possesses a highly modified dorsal petal (lip) [1], making them have high market needs and causing them to be removed from natural habitats. Moreover, some orchid seeds are hard to germinate and have a high mortality rate [2]. As a result of these, many species are at risk of extinction.

E. bicallosa is a terrestrial orchid that possesses beautiful white purplish flowers. The flowering period occurs during April and December. It can be found in southeast Asia (India, Indonesia, Malaysia, Myanmar, Nepal, New Guinea, Thailand, and Australia) [3]. It has a high potential to become a commercial, becoming more popular in local markets due to its beautiful flowers, therefore it raises a concern that this orchid species may become a threatened species soon.

Orchid seeds are hard to germinate in a natural environment [2]. The *in-vitro* seed germination protocol is a powerful tool for orchid germination, that is reported to be successful in many orchid species, such as *Calanthe* hybrids

("Hyesung" x "Jeongmong", "Hwagung" x "Heysung") [4], *Habenaria macroceratitis* [5], *Spathoglottis plicata* [6], *Eulophia nuda* [7], *Liparis fujisanensis*, *L. koreojaponica* and *L. kumokiri* [8].

The objective of this study was to investigate seed characteristics and identify suitable sterilizing and culture media for seed germination of *E. bicallosa* through asymbiotic germination.

2. Experiment

2.1 Seed source and sterilization

For seed source, *E. bicallosa* capsules and seeds were collected from Rayong Botanical Garden, Rayong Province with a permit. Then, seed capsules were stored in paper bags and dried on silica gel until the dehiscence. The brown seeds were then stored in a sterile Eppendorf tube at 4 ℃ (12% Humidity) until use.

To calculate seed weight and maturity, 1.2 mg of *E. bicallosa* seeds were weighed and counted. The seeds with fully developed embryos were counted as mature seeds. The length and width of randomized 30 seeds were measured.

For seed sterilization treatment, three concentrations (0, 0.5, 1%) and three durations (1, 5, 10 min) of commercial bleach (Hyter, 6% NaClO) were employed. After sterilization treatment, sterilized seeds were rinsed with deionized water for 5 min, 3 times before sawing. Seed viability using triphenyl tetrazolium chloride (TTC) staining (1%) was evaluated, using a microscope (Fig. 2A). After that approximately 150-200 seeds were sown onto each plate. Four replicate plates were used for each treatment. Seed germination percentage and contamination were recorded at one month, using stereoscope Fig. 2B-D and Fig. 3.

2.2 Effect of different media on seed germination

Seed germination and protocorm development were assessed by spreading seeds on six different types of culture media: (1) Malmgren Modified Terrestrial Orchid Medium (MM) [9], (2) half-strength MM (½MM), (3) Murashige and Skoog (MS) [10], (4) half-strength MS (½MS), (5) Vacin and Went (VW) [11], and (6) half-strength VW (½VW). The pH of all media was adjusted before sterilization and autoclaving at 121°C for 20 min. Sterilized seeds with the best sterilizing condition, 0.5% NaClO for 5 min, were sown on these media for 4 weeks under 0/24 h L/D and for 8 weeks under 16/8 h L/D photoperiod at 25 ± 2C. Approximately 150-200 seeds were sown onto each plate. Five replicate plates were used for each germination medium treatment. Percentages of seeds were recorded every month for three months. Protocorm development was scored on a scale of 0–5 [5].

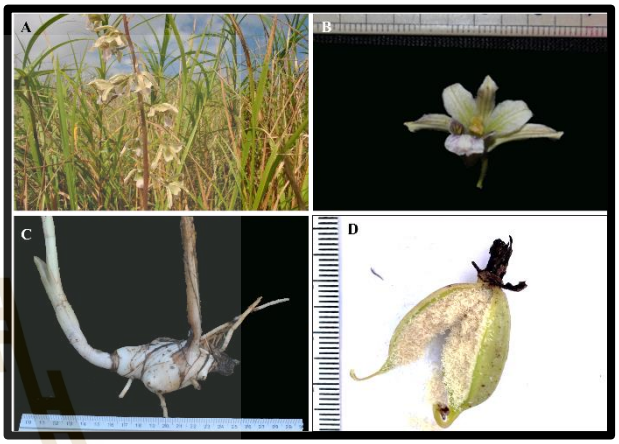

Fig. 1. Habit and inflorescence of *E. bicallosa.* A: habit; B: flower, C: rhizome, D: capsule and seeds.

3. Analysis

3.1 Statistic analysis

For the experiments, seed germination and protocorm development were observed every month for 3 months under a stereomicroscope. According to Stewart and Kane (2006), the germination and developmental stages were graded on a 0-5 scale of incremental growth. Percentages of seed germination and protocorms for each developmental stage were calculated from the total number of viable (maturation) seeds. All the experiments were conducted in a completely randomized design (CRD). Before data processing, the arcsine square root transformation was used to normalize variability. With the SPSS V26.0 statistical software (SPSS Inc., Chicago, USA), an analysis of variance (ANOVA) was performed, and the means were compared using the least significant difference (LSD) test (P=0.05).

4. Results and discussion

4.1 Seed characteristics and effect of NaClO on seed viability and germination

Like many other orchids, *E. bicallosa* seed is very tiny, dust-like, without endosperm, weighing about 4.50±0.29 µg. The seed length and width are about 628.32±9.15 µm and 195.69±4.15 µm respectively, while the embryo length and width are approximately 206.18±0.70 µm, and 117.16±3.76 µm, respectively (Table 1).

Embryos of the viable seeds of *E. bicallosa* stained bright red with 1% TTC, showing the high quality of the seed batches (66.90%) (Fig. 2A). After sterilization, seed viability with TTC showed variable among treatments (Table 1). The concentrations of 0.5% NaClO for 5 min (0.5x5) and 0.5% NaClO for 10 min (0.5x10) treatments gave moderate seed viability (62.06%, 59.10% respectively), whereas 0.5x1 and 1x1 treatments provided much higher percent seed viability (91.95%, and 87.31% respectively). NaClO for 1x 5 and 1x10 treatments had the lowest seed viability (13.82%, and 4.7% respectively). It is indicated that a shorter soaking time with NaClO gave more effectiveness on seed viability. After one month of sawing, some treatments got contaminated. Seed germination results are similar to seed maturity percentages, with 0.5x5 and 0.5x10 treatments providing a moderate percentage of seed germination (58.89%, and 58.42% respectively) without contamination. The 0.5x1 and 1x1 treatments gave the significantly highest percentage of seed germination (91.70%, and 83.52% respectively), but some got contaminated. The 1x5 and 1x10 treatments had the lowest seed germination percentages (21.14% and 1.49% respectively).

Without sterilant (0x10 treatment), all the samples got contaminated within the first week of sawing. Sterilization solution NaClO not only eliminated microbe from the tissue but also caused damage [12] and created stress to the tissue, stimulated metabolism that involved cell damage repair, and resulted in increased respiration. As a result of this, NaClO-treated seeds (0.5x1 and 1x1 treatments) were easier to be detected by TTC staining than untreated seeds (0x10). However, if NaClO is used with high concentration or long-duration treatment, it can cause decreasing in seed viability and germination due to damaged tissue which was noticeable, when compared to 0.5x5 and 0.5x10 treatments. With too low concentration or too short duration of NaClO, some microbe remained in the tissue, resulting in contamination during tissue culturing (0x10, 0.5x1, and 1x1 treatments).

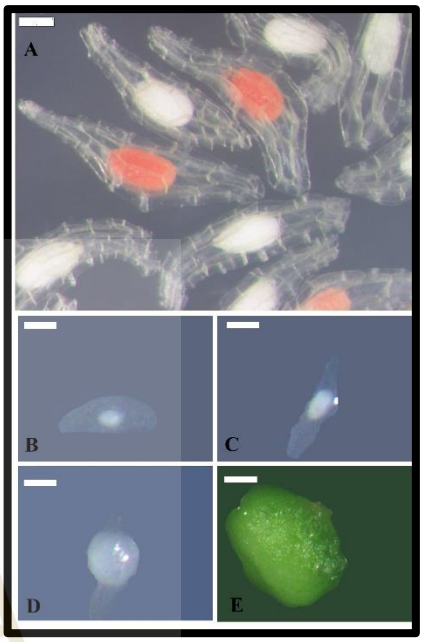

Fig. 2. TTC viability, and seed development A; treated seeds with 1% TTC (red= viability), B: stage 0, C: stage 1, D: stage 2; E: Stage 3 (scale bar= 100 µm).

Table 1. Seed characteristics of *E. bicallosa*.

Characteristics	Average ±SE
Seed weight	4.50 ±0.29 µg
Seed length (SL)	628.32±9.15 µm
Seed width (SW)	195.68±4.15 µm
SL/SW ratio	3.27
Embryo length (EL)	206.18±0.70 µm
Embryo width (EW)	117.16±3.76 µm
EL/EW ratio	1.79
Seed viability	66.90%

Table 2. Effect of NaClO concentration and duration on seed viability and germination.

Concentration (%) x Time (min)	Viability (%)	Germination (%)	Contami-nation
0 x 10	66.90 [a]	nd	++++
0.5 x 1	91.95 [b]	91.70 [a]	+
0.5 x 5	62.06 [a]	58.89 [b]	-
0.5 x10	59.10 [a]	58.42 [b]	-
1 x 1	87.37 [b]	83.52 [a]	+
1 x 5	13.82 [c]	21.14 [c]	+
1 x 10	4.70 [c]	1.49 [c]	-

Note: Different letters indicate significant differences between each treatment tested according to LSD test ($p < 0.05$). nd=not determine.

4.2 Effect of different media on seed germination

From three months of observation, development stages can be separated into 3 stages. At stage 1, seeds had not yet germinated, the testa intact with embryo length and width around 206.16±0.70 μm and 117.16±3.76 μm. Stage 2 seeds germinated with testa rupture due to an increase in the size of the embryo, in which some embryos are free from testa, embryos length and width around 378.33± 47.01 μm, and 303.78±40.95 μm. In stage 3, embryos are turning green and increase in size, embryo length and width are around 705±109.1 μm (twice larger) and 687.34±11.46 μm (6 times larger) (Fig. 2). This stage was noticeable in the second and third month of culture.

According to our findings, the kind and concentration of the basal media altered protocorm growth response. During three months of growth, all tested conditions sustained protocorm development (Fig. 3) to stage 3 protocorm. The rates of seed germination and protocorm development, however, differed between treatments (Table 3 and Fig. 3). MS (92.88 7.02%) and ½ MS (89.21 2.41%) had the highest seed germination rate and the highest proportion of stage 3 protocorms at 3 months after seed sawing compared to MM, ½ MM, VW, and 1/2 VW (Table 3). Protocorms grown in MM and ½ MM medium had well root development that could be due to the effect of activated charcoal in the medium.

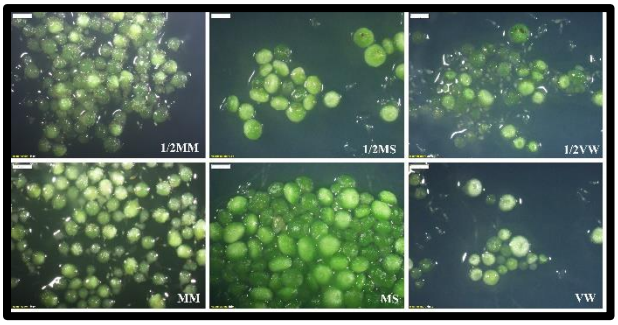

Fig. 3. Seed germination on various media at 3 months after sawing (scale bar= 1 mm).

Table 3. Seed germination stages of *E. bicallosa* on various media for 3 months. Different letters indicate significant differences in each media tested according to LSD test ($p < 0.05$).

Medium	Stage 0	Stage 1	Stage 2	Stage 3	Total germination
MM	29.32± 9.3[a]	0.57± 0.52[a]	9.22± 1.00[a]	60.88± 10.08[b]	70.67 ± 9.38[b]
½ MM*	19.88± 0.10	0.00	2.21 ± 1.10	77.91 ± 0.10	80.13 ± 1.00
MS	8.90± 6.68[b]	0.00	1.52 ± 0.43[b]	91.40 ± 8.60[a]	92.88 ± 7.02[a]
½ MS	10.79± 2.41[b]	0.00	0.00	89.21 ± 2.41[a]	89.21 ± 2.41[a]
VW	43.88± 9.68[a]	0.82 ± 0.42[a]	3.63 ± 3.31[ab]	49.70 ± 4.81[b]	56.12 ± 9.68[b]
½ VW	34.49± 4.24[a]	2.65 ± 2.18a	3.20 ± 2.97[ab]	65.51 ± 4.24[b]	65.51 ± 4.24[b]

½ MM *had only two replicate plates, unable to perform statistical analysis on this treatment*

The culture medium employed had a significant impact on germination due to the composition variation of organic and inorganic nutrients. The high germination and robust subsequent growth in MS and ½ MS media might be related to the fact that these media are particularly rich in both macro- and micronutrients when compared to MM and VW. It has previously been demonstrated that a high nitrogen supply affects the germination of many orchid species [5], [13], and [14]. Furthermore, orchids have been observed to use a species-specific medium for seed germination. Even within the same genus, medium variations have been found, such as BM1 for *Eulophia nuda* [6] and P723 medium for *Eulophia alta* [15], for *E. promensis* [16]. Using asymbiotic methods have been successfully reported in many *Eulophia* species, such as *E. cucullata*, *E. petersii*, and *E.*

streptopetala were all cultured on MS [17]. Because no plant growth regulators were added in this study, it suggests that sufficient endogenous hormones are already present.

5. Conclusions

Seed treatments with 0.5% NaClO for 5 min or 10 min resulted in moderate seed viability and germination of 58.89% and 58.42%, respectively, without contamination, thus is suitable for this orchid species. MS and ½ MS medium were observed to increase protocorm development in *E. bicallosa*, resulting in the highest seed germination and the largest proportion of Stage 3 protocorms at three months following seed sowing. This is the first report of *in vitro* germination of *E. bicallosa* seeds, which might be applied to other commercially significant, rare species, and endangered orchids for multiplication and conservation.

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References

[1] Taylor, T.N., Taylor, E.L., and Krings, M. PALEOBOTANY The Biology and Evolution of Fossil Plants (P. Gonzalez & A. Richford, Eds.; 2nd ed.). Academic Press, 2009.

[2] Kauth, P.J., Dutra, D., Johnson, T.R., Stewart, S.L., Kane, M.E., and Vendrame, W. Techniques and applications of *in vitro* orchid seed germination. Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues, 5, 375-391, 2008.

[3] Chen, X., Liu, Z., Zhu, G., Lang, K., Ji, Z., Luo, Y., and Bell, A. *Eulophia bicallosa*. Flora of China, 25, 253-254, 2009. Retrieved from http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=242321170

[4] Baque, M. A., Paek, K. Y., Shin, Y.K., Abdullahil Baque, M., Elghamedi, S., Lee, E.-J., and Paek, K.-Y. Effects of activated charcoal, plant growth regulators and ultrasonic pre-treatments on *in vitro* germination and protocorm formation of Calanthe hybrids. Australian Journal of Crop Science, 5(5), 582–588, 2011.

[5] Stewart, S.L., and Kane, M.E. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell, Tissue and Organ Culture, 86(2), 147–158, 2006.

[6] Nanekar, V., Shriram, V., Kumar, V., and Kishor, P. B. K. Asymbiotic *in vitro* seed germination and seedling development of *Eulophia nuda* Lindl., An endangered medicinal orchid. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 84(3), 837–846, 2014.

[7] Bran Barrientos, B. A., and Fang, J. Y. Influence of photoperiod and culture medium on the speed of asymbiotic seed germination and seedling development in *Spathoglottis plicata*. HortScience, 54(9), 1570–1575, 2019.

[8] Tsutsumi, C., Miyoshi, K., Yukawa, T., and Kato, M. Responses of seed germination and protocorm formation to light intensity and temperature in epiphytic and terrestrial *liparis* (Orchidaceae). Botany, 89(12), 841–848, 2011.

[9] Malmgren, S. Orchid propagation: theory and practice. In: Allen C (ed) North American native terrestrial orchids: propagation and production. North American Native Terrestrial Orchid Conference, Maryland. 63–71, 1996.

[10] Murashige, T., and Skoog, F. A. Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plantarum. 15: 473-497, 1962.

[11] Vacin, E.F., and Went, F.W. Some pH changes in nutrient solutions. Botanical Gazette110: 605–613, 1949.

[12] Yildiz, M., Ozcan, S.F., Kahramanogullari, C.T., and Tuna, E. The effect of sodium hypochlorite solutions on the viability and *in vitro* regeneration capacity of the tissue. The Natural Products Journal, 2(4), 328–331, 2012.

[13] Anderson, A.B. The reintroduction of *Platanthera ciliaris* in Canada; North American Native Terrestrial Orchid Conference; Germantown, MD. pp. 73–76.1996.

[14] Paul, S., Kumaria, S., and Tandon, P. An effective nutrient medium for asymbiotic seed germination and large-scale *in vitro* regeneration of *Dendrobium hookerianum*, a threatened orchid of northeast India. AoB Plants. 2012;2012:plr032. doi: 10.1093/aobpla/plr032. Epub 2011 Dec 22. PMCID: PMC3260561.

[15] Johnson, T. R., Stewart, S. L., Dutra, D., Kane, M. E., and Richardson, L. Asymbiotic and symbiotic seed germination of *Eulophia alta* (Orchidaceae)—preliminary evidence for the symbiotic culture advantage. Plant Cell, Tissue and Organ Culture, 90(3), 313–323, 2007.

[16] Hossain, MM. Ex vitro seedling development from *in vitro* rhizome-like bodies in *Eulophia promensis* Lindl.: A New Technique for orchid propagation. Journal of Botany.Volume 2015, Article ID 207694, http://dx.doi.org/10.1155/2015/207694

[17] McAlister, B.G., and Van Staden, J. *In vitro* culture of *Eulophia* species. S Afr J Bot 64:264–266, 1998.

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APPENDIX B

MEDIA AND COLCHICINE PREPARATION

1. Media preparation

1.1 MM and 1/2MM media were prepared using M534 Malmgren Modified Terrestrial Orchid Medium Without Sucrose and Agar (PhytoTechnology Laboratories ®, USA) according to product description. To prepare 1 liter MM medium; dissolve 21.84 g medium powder and 10 g sucrose in 1 liter DI water then adjust PH to 5.5-6 using NaOH and HCl, add 6 g agar and autoclave at 121 C, 15 PSI for 20 min. For 1/2MM, the method is the same as above except for medium powder and sucrose which are reduced to 10.92 g and 5 g respectively.

1.2 MS and 1/2MS media were prepared using M5800 Murashige and Skoog (MS) Modified Basal Medium (PhytoTechnology Laboratories ®, USA) according to product description. To prepare 1 liter MS; dissolve 4.406 g medium powder and 30 g sucrose in 1 liter DI water then adjust PH to 5.7-5.8 using NaOH and HCl, add 6 g agar and autoclave at 121 ^{O}C, 15 PSI for 20 min. For 1/2MS, the method is the same as above except for medium powder and sucrose which are reduced to 2.203 g and 15 g respectively.

1.3 VW and 1/2VW were prepared from stock solution A, B, and C. To prepare 1 liter VW; pipette 10 ml stock solution A, B, and C into 500 ml DI water, add 20 g sucrose, adjust volume to 1 liter using DI water, adjust PH to 4.8-5.5 using NaOH and HCl, add 6 g agar and autoclave at 121 C, 15 PSI for 20 min. For 1/2MS, the method is the same as above except for stock solution A, B, and C which are reduced to 5 ml. For stock solution preparation, I will describe below.

1) 1-liter stock A preparation. Put 50 g $(NH_4)_2SO_4$, 25 g $MgSO_4*7H_2O$, 52.5 g KNO_3, 25 g KH_2PO_4, 0.75 g $MnSO_4*4H_2O$ into each 200 ml beaker and dissolve in 180 ml DI water separately. Put dissolved solution together in 1000 ml volumetric flask ordinally start from $(NH_4)_2SO_4$ to $MgSO_4*7H_2O$ to KNO_3 to KH_2PO_4 to $MnSO_4$ $*4H_2O$, adjust the volume to 1 liter. Store at 4 C away from light.

2) 1-liter stock B preparation. Dissolve 20 g $Ca_3(PO_4)_2$ in minimal amount of 10% (v/v) HCl. Put the dissolved $Ca_3(PO_4)_2$ in 1000 ml volumetric flask containing small amount of DI water and adjust the volume to 1 liter using DI water. Store at 4 C away from light.

3) 1-liter stock C preparation. Dissolve 2.78 g $FeSO_4*7H_2O$ and 3.73 g Na_2EDTA in minimal amount of 50 C DI water separately. Put dissolved $FeSO_4*7H_2O$ into 1000 ml beaker containing small amount of DI water followed by dissolved Na_2EDTA, mix together, put into 1000 ml volumetric flask containing, and adjust volume to 1 liter. Store at 4 C away from light (use within 3 months).

2. Colchicine preparation

2.1 Prepare 2 ml of 0.2% (w/v) concentration colchicine stock solution by dissolving 0.004g colchicine in 2 ml DI water. Wrap with aluminum foil store at 4 C, away from light.

2.2 2 ml of 0.025, 0.05, 0.1 (w/v) colchicine were prepared from 0.2% (w/v) concentration colchicine stock solution. For 2 ml of 0.025 colchicine, pipette 0.25 ml colchicine stock solution into 5 ml vial bottle, and adjust the volume to 2 ml. For 2 ml of 0.05 (w/v) colchicine, pipette 0.5 ml colchicine stock solution into 5 ml vial bottle, and adjust the volume to 2 ml. For 2 ml of 0.1 (w/v) colchicine, pipette 0.1 ml colchicine stock solution into 5 ml vial bottle, and adjust the volume to 2 ml.

2.3 All colchicine vial were wrapped with aluminum foil and autoclave at at 121 °C, 15 PSI for 20 min. Leave at room temperature for 24 hours, away from the light and will be use within 48 hours after prepared.

APPENDIX C

RAW DATA

Table 6.1 Effect of Na(OCl) treatments on seed viability.

Na(OCl) Concentration (%)	Duration (min)	Replicate	Living seeds	Dead seeds	Unfertilized seeds	Fertilized seed	Total seeds
0	10	1	146	100	4	246	250
0	10	2	74	12	1	86	87
0	10	3	96	47	0	143	143
0	10	4	49	40	0	89	89
0.5	1	1	180	15	4	195	199
0.5	1	2	105	19	12	124	136
0.5	1	3	184	7	10	191	201
0.5	1	4	103	6	9	109	118
0.5	5	1	87	37	4	124	128
0.5	5	2	74	52	11	126	137
0.5	5	3	103	164	2	267	269
0.5	5	4	105	25	2	130	132

Table 6.1 Effect of Na(OCl) treatments on seed viability (Continued).

Na(OCl) Concentration(%)	Duration (min)	Replicate	Living seeds	Dead seeds	Unfertilized seeds	Fertilized seed	Total seeds
0.5	10	1	110	82	8	192	200
0.5	10	2	141	92	5	233	238
0.5	10	3	148	73	14	221	235
0.5	10	4	110	103	15	213	228
1	1	1	100	3	19	103	122
1	1	2	184	21	17	205	222
1	1	3	129	24	2	153	155
1	1	4	112	31	18	143	161
1	5	1	13	225	10	238	248
1	5	2	7	69	4	76	80
1	5	3	41	141	17	182	199
1	5	4	17	77	7	94	101
1	10	1	2	128	11	130	141
1	10	2	17	191	1	208	209
1	10	3	10	125	0	135	135
1	10	4	3	177	7	180	187

Table 6.2 Effect of Na(OCl) treatments on seed germination.

Na(OCl) Concentration(%)	Duration (min)	Replicate	germinated seeds	Total seeds
0	10	1	na	na
0	10	2	na	na
0	10	3	na	na
0	10	4	na	na
0.5	1	1	78	84
0.5	1	2	41	47
0.5	1	3	38	40
0.5	5	1	50	108
0.5	5	2	53	145
0.5	5	3	108	141
0.5	5	4	102	134

Remark: nd= no data (all sample are contaminated)

Table 6.2 Effect of Na(OCl) treatments on seed germination (Continued).

Na(OCl) Concentration(%)	Duration (min)	Replicate	germinated seeds	Total seeds
0.5	10	1	72	116
0.5	10	2	53	98
0.5	10	3	132	188
0.5	10	4	62	131
1	1	1	102	119
1	1	2	137	169
1	1	3	93	111
1	5	1	24	94
1	5	2	21	95
1	5	3	21	133
1	10	1	2	144
1	10	2	3	129
1	10	3	3	134
1	10	4	0	132

Table 6.3 influence of culture media on seed germination and development on the third month of culture.

Media	Replicate	Stage0	Stage1	Stage2	Stage3	Total
MM	1	73	2	16	101	192
MM	2	77	0	29	146	252
MM	3	57	2	23	212	294
1/2MM	1	32	0	5	130	167
1/2MM	2	43	0	3	163	209
MS	1	44	7	5	194	250
MS	2	0	0	0	155	155
MS	3	4	0	2	136	142
MS	4	11	0	0	93	104
MS	5	8	0	2	163	173
1/2MS	1	38	0	0	268	306
1/2MS	2	23	0	0	273	296
1/2MS	3	37	0	0	230	267
1/2MS	4	23	0	0	196	219
1/2MS	5	26	0	0	250	276

Table 6.3 influence of culture media on seed germination and development on the third month of culture (Continued).

Media	Replicate	Stage0	Stage1	Stage2	Stage3	Total
VW	1	57	30	17	95	199
VW	2	82	0	0	85	167
VW	3	91	1	4	85	181
VW	4	61	2	2	88	153
VW	5	84	1	4	74	163
1/2VW	1	97	0	3	167	267
1/2VW	2	92	16	20	154	282
1/2VW	3	57	1	11	134	203
1/2VW	4	109	6	7	177	299
1/2VW	5	97	6	0	146	249

Table 6.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment.

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	living protocorm	total protocorms
1	0	1	30	30
1	0	2	29	30
1	0	3	27	30
1	0	4	30	30
1	0	5	29	30
1	0	6	27	30
1	0.025	1	30	30
1	0.025	2	30	30
1	0.025	3	30	30
1	0.025	4	30	30
1	0.025	5	30	30
1	0.025	6	30	30

Table 6.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	living protocorm	total protocorms
1	0.05	1	29	30
1	0.05	2	29	30
1	0.05	3	30	30
1	0.05	4	29	30
1	0.05	5	29	30
1	0.05	6	30	30
1	0.1	1	26	30
1	0.1	2	26	30
1	0.1	3	30	30
1	0.1	4	26	30
1	0.1	5	26	30
1	0.1	6	30	30

Table 6.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	living protocorm	total protocorms
2	0	1	28	30
2	0	2	25	30
2	0	3	24	30
2	0	4	27	30
2	0	5	26	30
2	0	6	25	30
2	0.025	1	30	30
2	0.025	2	30	30
2	0.025	3	30	30
2	0.025	4	30	30
2	0.025	5	30	30
2	0.025	6	30	30

Table 6.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	living protocorm	total protocorms
2	0.05	1	29	30
2	0.05	2	29	30
2	0.05	3	29	30
2	0.05	4	29	30
2	0.05	5	29	30
2	0.05	6	29	30
2	0.1	1	26	30
2	0.1	2	26	30
2	0.1	3	29	30
2	0.1	4	26	30
2	0.1	5	26	30
2	0.1	6	27	30

Table 6.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	living protocorm	total protocorms
3	0	1	28	30
3	0	2	25	30
3	0	3	24	30
3	0	4	26	30
3	0	5	16	30
3	0	6	20	30
3	0.025	1	30	30
3	0.025	2	30	30
3	0.025	3	30	30
3	0.025	4	30	30
3	0.025	5	30	30
3	0.025	6	30	30

Table 6.4 Survival rate of *E. bicallosa* at 1, 2, and 3 months after colchicine treatment (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	living protocorm	total protocorms
3	0.05	1	29	30
3	0.05	2	29	30
3	0.05	3	29	30
3	0.05	4	29	30
3	0.05	5	29	30
3	0.05	6	29	30
3	0.1	1	26	30
3	0.1	2	25	30
3	0.1	3	29	30
3	0.1	4	26	30
3	0.1	5	26	30
3	0.1	6	27	30

Table 6.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa.*

month (after colchicine treatment)	colchicine concentration (%)	Replicate	stage 3 protocorm	stage 4 protocorm	stage 5 protocorm	rhizome-like bodies protocorm
1	0	1	28	2	0	0
1	0	2	29	0	0	0
1	0	3	27	0	0	0
1	0	4	30	0	0	0
1	0	5	29	0	0	0
1	0	6	27	0	0	0
1	0.025	1	30	0	0	0
1	0.025	2	27	2	1	0
1	0.025	3	22	6	2	0
1	0.025	4	30	0	0	0
1	0.025	5	26	4	0	0

Table 6.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa* (Continued).

month (after colchicine treatment)	colchicine concentration (%)	Replicate	stage 3 protocorm	stage 4 protocorm	stage 5 protocorm	rhizome-like bodies protocorm
1	0.025	6	20	9	1	0
1	0.05	1	20	9	0	0
1	0.05	2	20	9	0	0
1	0.05	3	27	2	1	0
1	0.05	4	29	0	0	0
1	0.05	5	26	3	0	0
1	0.05	6	27	2	1	0
1	0.1	1	23	3	0	0
1	0.1	2	24	2	0	0
1	0.1	3	30	0	0	0
1	0.1	4	26	0	0	0
1	0.1	5	26	0	0	0
1	0.1	6	30	0	0	0

Table 6.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa* (Continued).

month (after colchicine treatment)	colchicine concentration (%)	Replicate	stage 3 protocorm	stage 4 protocorm	stage 5 protocorm	rhizome-like bodies protocorm
2	0	1	0	0	0	28
2	0	2	0	0	0	25
2	0	3	0	0	0	24
2	0	4	0	0	0	27
2	0	5	25	1	0	0
2	0	6	23	2	0	0
2	0.025	1	0	0	0	30
2	0.025	2	0	0	0	30
2	0.025	3	0	0	0	30
2	0.025	4	0	0	0	30
2	0.025	5	0	0	0	30
2	0.025	6	0	0	0	30

Table 6.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa* (Continued).

month (after colchicine treatment)	colchicine concentration (%)	Replicate	stage 3 protocorm	stage 4 protocorm	stage 5 protocorm	rhizome-like bodies protocorm
2	0.05	1	0	0	0	29
2	0.05	2	0	0	0	29
2	0.05	3	0	0	0	29
2	0.05	4	2	0	0	27
2	0.05	5	0	0	0	29
2	0.05	6	0	0	0	29
2	0.1	1	0	0	0	26
2	0.1	2	0	0	0	26
2	0.1	3	0	0	0	29
2	0.1	4	1	0	0	25
2	0.1	5	0	0	0	26
2	0.1	6	2	0	0	25

Table 6.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa* (Continued).

month (after colchicine treatment)	colchicine concentration (%)	Replicate	stage 3 protocorm	stage 4 protocorm	stage 5 protocorm	rhizome-like bodies protocorm
3	0	1	0	0	0	28
3	0	2	0	0	0	25
3	0	3	0	0	0	24
3	0	4	0	0	0	26
3	0	5	0	0	0	16
3	0	6	0	0	0	20
3	0.025	1	0	0	0	30
3	0.025	2	0	0	0	30
3	0.025	3	0	0	0	30
3	0.025	4	0	0	0	30
3	0.025	5	0	0	0	30
3	0.025	6	0	0	0	30

Table 6.5 Effect of colchicine treatment on protocorm developmental stages of *E. bicalossa* (Continued).

month (after colchicine treatment)	colchicine concentration (%)	Replicate	stage 3 protocorm	stage 4 protocorm	stage 5 protocorm	rhizome-like bodies protocorm
3	0.05	1	0	0	0	29
3	0.05	2	0	0	0	29
3	0.05	3	0	0	0	29
3	0.05	4	0	0	0	29
3	0.05	5	0	0	0	29
3	0.05	6	0	0	0	29
3	0.1	1	0	0	0	26
3	0.1	2	0	0	0	25
3	0.1	3	0	0	0	29
3	0.1	4	0	0	0	26
3	0.1	5	0	0	0	26
3	0.1	6	0	0	0	27

Table 6.6 influence of colchicine treatment on shoot induction.

month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 shoot	Plant with 2 shoots	Plant with 3 shoots	Plant with 4 shoots
1	0	1	0	0	0	0
1	0	2	0	0	0	0
1	0	3	0	0	0	0
1	0	4	0	0	0	0
1	0	5	0	0	0	0
1	0	6	0	0	0	0
1	0.025	1	0	0	0	0
1	0.025	2	4	0	0	0
1	0.025	3	6	0	0	0
1	0.025	4	0	0	0	0
1	0.025	5	4	0	0	0
1	0.025	6	6	0	0	0

Table 6.6 influence of colchicine treatment on shoot induction (Continued).

month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 shoot	Plant with 2 shoots	Plant with 3 shoots	Plant with 4 shoots
1	0.05	1	0	0	0	0
1	0.05	2	2	1	0	0
1	0.05	3	2	0	0	0
1	0.05	4	0	0	0	0
1	0.05	5	2	1	0	0
1	0.05	6	2	0	0	0
1	0.1	1	0	0	0	0
1	0.1	2	0	0	0	0
1	0.1	3	0	0	0	0
1	0.1	4	0	0	0	0
1	0.1	5	0	0	0	0
1	0.1	6	0	0	0	0

Table 6.6 influence of colchicine treatment on shoot induction (Continued).

month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 shoot	Plant with 2 shoots	Plant with 3 shoots	Plant with 4 shoots
2	0	1	0	0	0	0
2	0	2	0	0	0	0
2	0	3	0	0	0	0
2	0	4	0	0	0	0
2	0	5	1	0	0	0
2	0	6	2	1	0	1
2	0.025	1	3	1	0	0
2	0.025	2	5	2	0	0
2	0.025	3	1	1	0	0
2	0.025	4	6	0	0	0
2	0.025	5	2	3	0	0
2	0.025	6	7	2	0	0

Table 6.6 influence of colchicine treatment on shoot induction (Continued).

month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 shoot	Plant with 2 shoots	Plant with 3 shoots	Plant with 4 shoots
2	0.05	1	1	1	0	0
2	0.05	2	0	1	0	0
2	0.05	3	2	0	0	0
2	0.05	4	7	2	0	0
2	0.05	5	3	0	1	0
2	0.05	6	5	0	1	0
2	0.1	1	0	0	0	0
2	0.1	2	0	0	0	0
2	0.1	3	1	0	0	0
2	0.1	4	6	0	0	0
2	0.1	5	0	0	0	0
2	0.1	6	0	0	0	0

Table 6.6 influence of colchicine treatment on shoot induction (Continued).

month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 shoot	Plant with 2 shoots	Plant with 3 shoots	Plant with 4 shoots
3	0	1	1	0	0	0
3	0	2	0	0	0	0
3	0	3	0	0	0	0
3	0	4	0	0	0	0
3	0	5	0	0	0	0
3	0	6	1	1	0	0
3	0.025	1	6	3	0	0
3	0.025	2	8	1	0	0
3	0.025	3	1	1	0	0
3	0.025	4	11	0	0	0
3	0.025	5	8	2	0	0
3	0.025	6	10	3	0	0

Table 6.6 influence of colchicine treatment on shoot induction (Continued).

month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 shoot	Plant with 2 shoots	Plant with 3 shoots	Plant with 4 shoots
3	0.05	1	3	2	0	0
3	0.05	2	13	0	0	0
3	0.05	3	5	2	1	0
3	0.05	4	17	1	2	0
3	0.05	5	8	0	0	0
3	0.05	6	8	1	1	0
3	0.1	1	2	0	0	0
3	0.1	2	4	1	0	0
3	0.1	3	2	0	0	0
3	0.1	4	6	3	0	0
3	0.1	5	5	1	0	0
3	0.1	6	6	0	0	0

Table 6.7 influence of colchicine treatment on root induction.

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 root	Plant with 2 roots	Plant with 3 roots	Plant with 4 roots
1	0	1	0	0	0	0
1	0	2	0	0	0	0
1	0	3	0	0	0	0
1	0	4	0	0	0	0
1	0	5	0	0	0	0
1	0	6	0	0	0	0
1	0.025	1	0	0	0	0
1	0.025	2	0	0	0	0
1	0.025	3	1	0	0	0
1	0.025	4	0	0	0	0
1	0.025	5	0	0	0	0
1	0.025	6	1	0	0	0

Table 6.7 influence of colchicine treatment on root induction (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 root	Plant with 2 roots	Plant with 3 roots	Plant with 4 roots
1	0.05	1	0	0	0	0
1	0.05	2	0	0	0	0
1	0.05	3	1	0	0	0
1	0.05	4	0	0	0	0
1	0.05	5	0	0	0	0
1	0.05	6	0	0	0	0
1	0.1	1	0	0	0	0
1	0.1	2	0	0	0	0
1	0.1	3	0	0	0	0
1	0.1	4	0	0	0	0
1	0.1	5	0	0	0	0
1	0.1	6	0	0	0	0

Table 6.7 influence of colchicine treatment on root induction (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 root	Plant with 2 roots	Plant with 3 roots	Plant with 4 roots
2	0	1	0	0	0	0
2	0	2	0	0	0	0
2	0	3	0	0	0	0
2	0	4	0	0	0	0
2	0	5	0	0	0	0
2	0	6	0	0	0	0
2	0.025	1	4	1	0	0
2	0.025	2	0	2	0	0
2	0.025	3	0	0	0	0
2	0.025	4	1	5	0	0
2	0.025	5	3	3	1	0
2	0.025	6	3	0	1	1

Table 6.7 influence of colchicine treatment on root induction (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 root	Plant with 2 roots	Plant with 3 roots	Plant with 4 roots
2	0.05	1	0	2	0	0
2	0.05	2	2	2	0	0
2	0.05	3	3	0	0	0
2	0.05	4	4	1	0	0
2	0.05	5	3	1	0	0
2	0.05	6	4	3	1	0
2	0.1	1	0	0	0	0
2	0.1	2	0	0	0	0
2	0.1	3	1	0	0	0
2	0.1	4	3	1	0	0
2	0.1	5	3	0	0	0
2	0.1	6	3	0	0	0

Table 6.7 influence of colchicine treatment on root induction (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 root	Plant with 2 roots	Plant with 3 roots	Plant with 4 roots
3	0	1	0	0	0	0
3	0	2	0	0	0	0
3	0	3	0	0	0	0
3	0	4	0	0	0	0
3	0	5	0	0	0	0
3	0	6	1	0	0	0
3	0.025	1	2	2	0	1
3	0.025	2	1	3	1	1
3	0.025	3	2	0	0	0
3	0.025	4	3	2	0	0
3	0.025	5	3	2	0	0
3	0.025	6	6	4	0	0

Table 6.7 influence of colchicine treatment on root induction (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant with 1 root	Plant with 2 roots	Plant with 3 roots	Plant with 4 roots
3	0.05	1	3	2	0	0
3	0.05	2	3	2	0	0
3	0.05	3	3	2	0	0
3	0.05	4	5	2	1	0
3	0.05	5	6	2	0	0
3	0.05	6	4	2	4	0
3	0.1	1	3	0	0	0
3	0.1	2	2	1	0	0
3	0.1	3	1	1	0	0
3	0.1	4	4	5	2	0
3	0.1	5	4	2	0	0
3	0.1	6	3	2	0	0

Table 6.8 Various shoot length due to the effect of colchicine.

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
1	0	1	no shoot	no shoot
1	0	2	no shoot	no shoot
1	0	3	no shoot	no shoot
1	0	4	no shoot	no shoot
1	0	5	no shoot	no shoot
1	0	6	no shoot	no shoot
1	0.025	1	no shoot	no shoot
1	0.025	2	1	0.3
1	0.025	2	2	0.2
1	0.025	2	3	0.2
1	0.025	2	4	0.2
1	0.025	3	1	0.7
1	0.025	3	2	0.2
1	0.025	3	3	0.4
1	0.025	3	4	0.3
1	0.025	3	5	0.2

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
1	0.025	3	6	0.2
1	0.025	4	no shoot	no shoot
1	0.025	5	1	0.3
1	0.025	5	2	0.2
1	0.025	5	3	0.2
1	0.025	5	4	0.2
1	0.025	6	1	0.7
1	0.025	6	2	0.2
1	0.025	6	3	0.4
1	0.025	6	4	0.3
1	0.025	6	5	0.2
1	0.025	6	6	0.2

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
1	0.05	1	no shoot	no shoot
1	0.05	2	1	0.4
1	0.05	2	2	0.3
1	0.05	2	3	0.3
1	0.05	3	1	0.7
1	0.05	3	2	0.3
1	0.05	4	no shoot	no shoot
1	0.05	5	1	0.4
1	0.05	5	2	0.3
1	0.05	5	3	0.3
1	0.05	6	1	0.7
1	0.05	6	2	0.3

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
1	0.1	1	no shoot	no shoot
1	0.1	2	no shoot	no shoot
1	0.1	3	no shoot	no shoot
1	0.1	4	no shoot	no shoot
1	0.1	5	no shoot	no shoot
1	0.1	6	no shoot	no shoot
2	0	1	no shoot	no shoot
2	0	2	no shoot	no shoot
2	0	3	no shoot	no shoot
2	0	4	no shoot	no shoot
2	0	5	1	0.7
2	0	6	1	1
2	0	6	2	1
2	0	6	3	1
2	0	6	4	9

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
2	0.025	1	1	1.8
2	0.025	1	2	3.46
2	0.025	1	3	1.21
2	0.025	1	4	0.71
2	0.025	2	1	2.4
2	0.025	2	2	2.06
2	0.025	2	3	1.28
2	0.025	2	4	1.14
2	0.025	2	5	0.82
2	0.025	2	6	1.21
2	0.025	2	7	0.51
2	0.025	3	1	0.86
2	0.025	3	2	1.39
2	0.025	4	1	1
2	0.025	4	2	1
2	0.025	4	3	1

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
2	0.025	4	4	0.5
2	0.025	4	5	0.2
2	0.025	4	6	0.2
2	0.025	5	1	0.5
2	0.025	5	2	0.5
2	0.025	5	3	2.5
2	0.025	5	4	2.5
2	0.025	5	5	0.3
2	0.025	6	1	1.5
2	0.025	6	2	4
2	0.025	6	3	1
2	0.025	6	4	2
2	0.025	6	5	5
2	0.025	6	6	5
2	0.025	6	7	1.5
2	0.025	6	8	1.2

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
2	0.025	6	9	5
2	0.05	1	1	1.44
2	0.05	1	2	0.76
2	0.05	2	1	2.07
2	0.05	2	2	4.37
2	0.05	2	3	1.58
2	0.05	2	4	2.07
2	0.05	3	1	1.72
2	0.05	3	2	0.64
2	0.05	4	1	1.5
2	0.05	4	2	0.2
2	0.05	4	3	0.2
2	0.05	4	4	1
2	0.05	4	5	1
2	0.05	4	6	0.5
2	0.05	4	7	0.5

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
2	0.05	4	8	0.5
2	0.05	4	9	0.5
2	0.05	5	1	4
2	0.05	5	2	1.5
2	0.05	5	3	1.5
2	0.05	5	4	3
2	0.05	6	1	9
2	0.05	6	2	2
2	0.05	6	3	2
2	0.05	6	4	2
2	0.05	6	5	4
2	0.05	6	6	2

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
2	0.1	1	no shoot	no shoot
2	0.1	2	no shoot	no shoot
2	0.1	3	1	1.51
2	0.1	4	1	1
2	0.1	4	2	0.5
2	0.1	4	3	0.5
2	0.1	4	4	0.5
2	0.1	4	5	0.5
2	0.1	4	6	0.3
2	0.1	5	no shoot	no shoot
2	0.1	6	no shoot	no shoot

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0	1	1	1.25
3	0	2	no shoot	no shoot
3	0	3	no shoot	no shoot
3	0	4	no shoot	no shoot
3	0	5	no shoot	no shoot
3	0	6	1	9
3	0	6	2	5
3	0.025	1	1	2.51
3	0.025	1	2	5.82
3	0.025	1	3	2.8
3	0.025	1	4	4.84
3	0.025	1	5	5.66
3	0.025	1	6	5.68
3	0.025	1	7	4.84
3	0.025	1	8	4.86
3	0.025	1	9	1.28

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.025	2	1	4.17
3	0.025	2	2	6.62
3	0.025	2	3	8.09
3	0.025	2	4	5.07
3	0.025	2	5	6.12
3	0.025	2	6	3.48
3	0.025	2	7	1.61
3	0.025	2	8	4.35
3	0.025	2	9	3.94
3	0.025	3	1	5.02
3	0.025	3	2	1.08
3	0.025	4	1	4
3	0.025	4	2	0.5
3	0.025	4	3	2
3	0.025	4	4	2.5
3	0.025	4	5	7

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.025	4	6	1
3	0.025	4	7	4
3	0.025	4	8	1
3	0.025	4	9	1
3	0.025	4	10	0.5
3	0.025	4	11	1
3	0.025	5	1	6
3	0.025	5	2	9
3	0.025	5	3	3
3	0.025	5	4	4
3	0.025	5	5	7
3	0.025	5	6	6
3	0.025	5	7	9
3	0.025	5	8	2
3	0.025	5	9	3
3	0.025	5	10	2

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.025	5	11	2
3	0.025	6	1	9
3	0.025	6	2	5
3	0.025	6	3	3
3	0.025	6	4	2
3	0.025	6	5	5
3	0.025	6	6	7
3	0.025	6	7	3
3	0.025	6	8	4
3	0.025	6	9	9
3	0.025	6	10	3
3	0.025	6	11	3
3	0.025	6	12	6
3	0.025	6	13	2

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.05	1	1	4.62
3	0.05	1	2	3.98
3	0.05	1	3	2.48
3	0.05	1	4	1.74
3	0.05	1	5	1.46
3	0.05	2	1	5.98
3	0.05	2	2	2.46
3	0.05	2	3	0.94
3	0.05	2	4	4.38
3	0.05	2	5	1.77
3	0.05	2	6	7.42
3	0.05	2	7	4.56
3	0.05	2	8	8.14
3	0.05	2	9	8.14
3	0.05	2	10	3.48
3	0.05	2	11	2.46

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.05	2	12	1.78
3	0.05	2	13	1.63
3	0.05	3	1	5.33
3	0.05	3	2	10.05
3	0.05	3	3	5.07
3	0.05	3	4	3.15
3	0.05	3	5	4.46
3	0.05	3	6	7.65
3	0.05	3	7	3.64
3	0.05	3	8	1.85
3	0.05	4	1	5
3	0.05	4	2	1
3	0.05	4	3	5
3	0.05	4	4	3
3	0.05	4	5	1
3	0.05	4	6	5

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.05	4	7	2
3	0.05	4	8	2
3	0.05	4	9	0
3	0.05	4	10	5
3	0.05	4	11	8
3	0.05	4	12	1
3	0.05	4	13	2
3	0.05	4	14	5
3	0.05	4	15	3
3	0.05	4	16	5
3	0.05	4	17	9
3	0.05	4	18	8
3	0.05	4	19	3
3	0.05	4	20	3

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.05	5	1	3
3	0.05	5	2	3
3	0.05	5	3	5
3	0.05	5	4	4
3	0.05	5	5	3
3	0.05	5	6	4
3	0.05	5	7	3
3	0.05	5	8	2
3	0.05	6	1	9
3	0.05	6	2	5
3	0.05	6	3	9
3	0.05	6	4	3
3	0.05	6	5	5
3	0.05	6	6	5
3	0.05	6	7	9
3	0.05	6	8	3

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.05	6	9	6
3	0.05	6	10	2
3	0.1	1	1	2.21
3	0.1	1	2	7.12
3	0.1	2	1	1.21
3	0.1	2	2	1.84
3	0.1	2	3	2
3	0.1	2	4	0.61
3	0.1	2	5	1.74
3	0.1	3	1	0.93
3	0.1	3	2	0.43
3	0.1	4	1	7
3	0.1	4	2	2
3	0.1	4	3	4
3	0.1	4	4	4
3	0.1	4	5	4

Table 6.8 Various shoot length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine concentration (%)	Replicate	Plant number	Length of the longest shoot
3	0.1	4	6	6
3	0.1	4	7	3
3	0.1	4	8	3
3	0.1	4	9	2
3	0.1	5	1	2
3	0.1	5	2	1
3	0.1	5	3	1
3	0.1	5	4	1
3	0.1	5	5	0.5
3	0.1	5	6	0.5
3	0.1	6	1	1.5
3	0.1	6	2	1.5
3	0.1	6	3	6
3	0.1	6	4	3
3	0.1	6	5	1
3	0.1	6	6	4

Table 6.9 Various root length due to the effect of colchicine.

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
1	0	1	no root	no root
1	0	2	no root	no root
1	0	3	no root	no root
1	0	4	no root	no root
1	0	5	no root	no root
1	0	6	no root	no root
1	0.025	1	no root	no root
1	0.025	2	no root	no root
1	0.025	3	1	1.1
1	0.025	4	no root	no root
1	0.025	5	no root	no root
1	0.025	6	1	1.1

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
1	0.05	1	no root	no root
1	0.05	2	no root	no root
1	0.05	3	1	0.2
1	0.05	4	no root	no root
1	0.05	5	no root	no root
1	0.05	6	1	0.2
1	0.1	1	no root	no root
1	0.1	2	no root	no root
1	0.1	3	no root	no root
1	0.1	4	no root	no root
1	0.1	5	no root	no root
1	0.1	6	no root	no root

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
2	0	1	no root	no root
2	0	2	no root	no root
2	0	3	no root	no root
2	0	4	no root	no root
2	0	5	no root	no root
2	0	6	no root	no root
2	0.025	1	1	0.73
2	0.025	1	2	0.76
2	0.025	1	3	0.68
2	0.025	1	4	0.57
2	0.025	1	5	0.49
2	0.025	2	1	1.54
2	0.025	2	2	1.13
2	0.025	3	no root	no root

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
2	0.025	4	1	1
2	0.025	4	2	1
2	0.025	4	3	1
2	0.025	4	4	0.5
2	0.025	4	5	0.5
2	0.025	4	6	0.5
2	0.025	5	1	0.5
2	0.025	5	2	0.5
2	0.025	5	3	1
2	0.025	5	4	0.5
2	0.025	5	5	0.5
2	0.025	5	6	0.5
2	0.025	5	7	1

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
2	0.025	6	1	1.5
2	0.025	6	2	1
2	0.025	6	3	1
2	0.025	6	4	1
2	0.025	6	5	1
2	0.05	1	1	0.65
2	0.05	2	1	0.82
2	0.05	2	2	0.81
2	0.05	2	3	1.03
2	0.05	2	4	0.93
2	0.05	3	1	1.16
2	0.05	3	2	0.73
2	0.05	3	3	0.53

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
2	0.05	4	1	1
2	0.05	4	2	2
2	0.05	4	3	1
2	0.05	4	4	1
2	0.05	4	5	1
2	0.05	5	1	0.4
2	0.05	5	2	0.2
2	0.05	5	3	0.2
2	0.05	5	4	0.3
2	0.05	6	1	1.2
2	0.05	6	2	0.5
2	0.05	6	3	1
2	0.05	6	4	1
2	0.05	6	5	1
2	0.05	6	6	1
2	0.05	6	7	1

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
2	0.05	6	8	1
2	0.1	1	no shoot	no shoot
2	0.1	2	no shoot	no shoot
2	0.1	3	1	0.47
2	0.1	4	1	1
2	0.1	4	1	0.3
2	0.1	4	2	1
2	0.1	4	3	0.5
2	0.1	5	1	0.3
2	0.1	5	2	0.3
2	0.1	5	3	0.3
2	0.1	6	1	0.3
2	0.1	6	2	0.3
2	0.1	6	3	0.3

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0	1	no root	no root
3	0	2	no root	no root
3	0	3	no root	no root
3	0	4	no root	no root
3	0	5	no root	no root
3	0	6	1	0.5
3	0	6	2	1
3	0.025	1	1	1.34
3	0.025	1	2	1.19
3	0.025	1	3	1.38
3	0.025	1	4	0.56
3	0.025	1	5	0.68

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.025	2	1	1.29
3	0.025	2	2	1.08
3	0.025	2	3	1.11
3	0.025	2	4	1.25
3	0.025	2	5	2.05
3	0.025	2	6	1.2
3	0.025	3	1	0.79
3	0.025	3	2	1.19
3	0.025	4	1	1.5
3	0.025	4	2	1.2
3	0.025	4	3	1.5
3	0.025	4	4	2
3	0.025	4	5	0.5

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.025	5	1	1
3	0.025	5	2	1
3	0.025	5	3	1
3	0.025	5	4	1.5
3	0.025	5	5	1
3	0.025	6	1	1
3	0.025	6	2	1
3	0.025	6	3	1
3	0.025	6	4	1
3	0.025	6	5	2
3	0.025	6	6	1.5
3	0.025	6	7	2
3	0.025	6	8	1.5
3	0.025	6	9	1
3	0.025	6	10	1

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.05	1	1	1.1
3	0.05	1	2	0.88
3	0.05	1	3	0.92
3	0.05	1	4	0.69
3	0.05	1	5	0.9
3	0.05	2	1	1.45
3	0.05	2	2	1
3	0.05	2	3	1.24
3	0.05	2	4	0.94
3	0.05	2	5	0.87
3	0.05	3	1	0.859
3	0.05	3	2	1.328
3	0.05	3	3	1.902
3	0.05	3	4	0.779
3	0.05	3	5	0.746

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.05	4	1	5
3	0.05	4	2	1.5
3	0.05	4	3	3
3	0.05	4	4	1.5
3	0.05	4	5	2
3	0.05	4	6	2
3	0.05	4	7	0.5
3	0.05	4	8	8
3	0.05	5	1	3
3	0.05	5	2	3
3	0.05	5	3	5
3	0.05	5	4	4
3	0.05	5	1	3
3	0.05	5	2	4
3	0.05	5	3	3
3	0.05	5	4	2

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.05	6	1	9
3	0.05	6	2	5
3	0.05	6	3	9
3	0.05	6	4	3
3	0.05	6	5	5
3	0.05	6	6	5
3	0.05	6	7	9
3	0.05	6	8	3
3	0.05	6	9	6
3	0.05	6	10	2
3	0.1	1	1	0.58
3	0.1	1	2	0.76
3	0.1	1	3	0.72

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.1	2	1	0.93
3	0.1	2	2	0.42
3	0.1	2	3	0.48
3	0.1	3	1	0.81
3	0.1	3	2	0.72
3	0.1	4	1	1
3	0.1	4	2	1
3	0.1	4	3	2
3	0.1	4	4	2.5
3	0.1	4	5	1
3	0.1	4	6	1
3	0.1	4	7	1
3	0.1	4	8	1
3	0.1	4	9	1.5
3	0.1	4	10	1.5
3	0.1	4	11	0.5

Table 6.9 Various root length due to the effect of colchicine (Continued).

Month (after colchicine treatment)	Colchicine Concentration (%)	Replicate	Plant number	Length of the longest root
3	0.1	5	1	1.5
3	0.1	5	2	0.5
3	0.1	5	3	0.5
3	0.1	5	4	1
3	0.1	5	5	1
3	0.1	5	6	0.5
3	0.1	6	1	3
3	0.1	6	2	0.5
3	0.1	6	3	1
3	0.1	6	4	1
3	0.1	6	5	0.5

APPENDIX D: *Eulophia* chromosomes

Table 6.10 Gametophyte chromosomes of *Eulophia* spp.

Species	Gametophyte chromosomes	Source
Eulophia aculeata subsp. huttonii (Rolfe) A. V. Hall	27	CCDB chromosome count data base
Eulophia angolensis (Rchb.) Summerh.	34	CCDB chromosome count data base
Eulophia angolensis (Rchb.) Summerh.	35	CCDB chromosome count data base
Eulophia angolensis (Rchb.) Summerh.	36	CCDB chromosome count data base
Eulophia angolensis (Rchb.) Summerh.	37	CCDB chromosome count data base
Eulophia angolensis (Rchb.) Summerh.	38	CCDB chromosome count data base
Eulophia campestris Wall.	24	IPCN chromosome report
Eulophia cristata (Afzel.) Steud.	23	CCDB chromosome count data base
Eulophia dabia (D. Don) Hochr.	24	CCDB chromosome count data base
Eulophia dabia (D. Don) Hochr.	27	CCDB chromosome count data base
Eulophia dentata Ames	27	CCDB chromosome count data base
Eulophia dentata Ames	28	CCDB chromosome count data base
Eulophia ensata Lindl.	27	CCDB chromosome count data base
Eulophia epidendraea (J. Koenig) C. E. C. Fisch.	27	CCDB chromosome count data base
Eulophia epidendraea C.E.C. Fisch.	27	IPCN chromosome report

Table 6.10 Gametophyte chromosomes of *Eulophia* spp. (Continued).

Species	Gametophyte chromosomes	Source
Eulophia euglossa (Rchb. f.) Rchb. f.	22	IPCN chromosome report
Eulophia euglossa (Rchb.) Rchb. ex Bateman	22	CCDB chromosome count data base
Eulophia euglossa (Rchb.) Rchb. ex Bateman	20	CCDB chromosome count data base
Eulophia exaltata Rchb.	16	CCDB chromosome count data base
Eulophia foliosa (Lindl.) Bolus	27	CCDB chromosome count data base
Eulophia fridericii (Rchb.) A. V. Hall	24	CCDB chromosome count data base
Eulophia gracilis Lindl	22	PROTA4U
Eulophia graminea Lindl.	28	CCDB chromosome count data base
Eulophia graminea Lindl.	27	CCDB chromosome count data base
Eulophia graminea Lindl.	21	CCDB chromosome count data base
Eulophia graminea Lindl.	24	CCDB chromosome count data base
Eulophia graminea Lindl.	22	CCDB chromosome count data base
Eulophia graminea Lindl.	23	CCDB chromosome count data base
Eulophia graminea Lindl.	48	CCDB chromosome count data base
Eulophia hians Spreng.	50	CCDB chromosome count data base
Eulophia hians var. nutans (Sond.) S. Thomas	25	CCDB chromosome count data base
Eulophia hians var. nutans (Sond.) S. Thomas	47	CCDB chromosome count data base

Table 6.10 Gametophyte chromosomes of *Eulophia* spp. (Continued).

Species	Gametophyte chromosomes	Source
Eulophia hormusjii A.V. Duthie	27	IPCN chromosome report
Eulophia horsfallii (Bateman) Summerh.	31	CCDB chromosome count data base
Eulophia leachii Greatrex ex A. V. Hall	26	CCDB chromosome count data base
Eulophia leontoglossa Rchb.	27	CCDB chromosome count data base
Eulophia macowanii Rolfe	28	CCDB chromosome count data base
Eulophia macrostachya Lindl.	17	IPCN chromosome report
Eulophia nuda Lindl.	27	IPCN chromosome report
Eulophia nuda var. andersonii Hook. f.	28	IPCN chromosome report
Eulophia ochreata Lindl.	27	CCDB chromosome count data base
Eulophia ovalis Lindl.	21	CCDB chromosome count data base
Eulophia ovalis Lindl.	40	CCDB chromosome count data base
Eulophia ovalis var. bainesii (Rolfe) P. J. Cribb & L. Croix	42	CCDB chromosome count data base
Eulophia paniculata Rolfe	30	IPCN chromosome report
Eulophia parviflora (Lindl.) A. V. Hall	25	CCDB chromosome count data base
Eulophia petersii (Rchb.) Rchb.	24	CCDB chromosome count data base
Eulophia promensis Lindl.	19	CCDB chromosome count data base
Eulophia pulchra (Thouars) Lindl.	16	CCDB chromosome count data base

Table 6.10 Gametophyte chromosomes of *Eulophia* spp. (Continued).

Species	Gametophyte chromosomes	Source
Eulophia pulchra (Thouars) Lindl.	17	CCDB chromosome count data base
Eulophia R. Br. ex Lindl.	27	IPCN chromosome report
Eulophia ramentacea Lindl.	27	IPCN chromosome report
Eulophia speciosa (R. Br.) Bolus	20	CCDB chromosome count data base
Eulophia speciosa (R. Br.) Bolus	27	CCDB chromosome count data base
Eulophia spectabilis (Dennst.) Suresh	16	CCDB chromosome count data base
Eulophia spectabilis (Dennst.) Suresh	27	CCDB chromosome count data base
Eulophia spectabilis (Dennst.) Suresh	16	CCDB chromosome count data base
Eulophia streptopetala Lindl.	42	CCDB chromosome count data base
Eulophia streptopetala Lindl.	21	CCDB chromosome count data base
Eulophia streptopetala Lindl.	20	CCDB chromosome count data base
Eulophia tenella Rchb.	60	CCDB chromosome count data base
Eulophia tuberculata Bolus	50	CCDB chromosome count data base
Eulophia welwitschii (Rchb.) Rolfe	27	CCDB chromosome count data base
Eulophia zeyheriana Sond.	56	CCDB chromosome count data base
Eulophia zollingeri (Rchb.) J. J. Sm.	17	CCDB chromosome count data base

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Publications:

Worapat Khankhokkruad, Santi Watthana, and Nooduan Muangsan. 2023.The Effect of Sodium Hypochlorite and Culture Medium on Seed Viability and Germination of *Eulophia bicallosa* (D. Don) Hunt & Summerh (Orchidaceae). PROCEEDINGS of the 17th SOUTH EAST ASIAN TECHNICAL UNIVERSITY CONSORTIUM, 23-24 February 2023. Thailand. Grants and Fellowships DPST Scholarships.