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Study on growth performance of *Rhododendron arboretum Sm.* by seed culture

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စာတမ်းအကျဉ်း

တောင်ဇလပ်သစ်မျိုးသည်ကြီးမားသောမျိုးစုဝင်သစ်မာမျိုးဖြစ်ပြီး Ericaceae မျိုးရင်းဝင် အပင်မျိုးဖြစ်၍ မျိုးစု တခုတွင် ၁၀၀၀ မျိုးစိတ်ပါဝင်၍ မျိုးခွဲ ၈ ခု ပါဝင်ပါသည်။ (Cham berlain et al., 1996). *Rhododendron arboreum* Sm. မျိုးစိတ်များသည် အစေ့မှ သဘာဝအတိုင်း ပေါက်ပွားပြီး မျိုးပွားနှုန်း နည်းပါသည်။ ထိုမျိုးစိတ်များကို ထိန်းသိမ်းစောင့်ရှောက်ရန်အတွက် မျိုးစေ့ပေါက်ခြင်းကို စူးစမ်းလေ့လာခြင်းသည် အရေးကြီးပါသည်။ ပိုးသတ်ခြင်းစမ်းသပ်မှုကို ပိုးသတ်ခြင်းနည်းစနစ်သုံးမျိုး (Sterilization A, B, C) သုံးကြိမ် ပြုလုပ်ပြီး စမ်းသပ်ပြွန်တစ်ခုတွင် အစေ့ ၄၀ ပါဝင်ပါသည်။ ပိုးသတ်ခြင်း A နှင့် ပိုးသတ်ခြင်း C ထက် ပိုးသတ်ခြင်း B (Homine +Tween 20– 15 minute, Kasumin + Clorox –10 minute, HgCl2+Tween20 – 5 second, double distilled water (DDW) – 3 times)သည် အကောင်းဆုံးဖြစ်ကြောင်း တွေ့ရှိခဲ့ရပါသည်။ Induction (စတင်မွေးမြူခြင်း) စမ်းသပ်မှုတွင် အာဟာရ မျိုးပွားစာ(Treatment) ၆ မျိုးဖြင့်သုံးကြိမ် စမ်းသပ်ခဲ့ပါသည်။ Treatment 4 (Murashige and Skoog (MS) without hormone,3% sucrose , 0.8% Agar) သည် အခြား Treatment များထက် ကြီးထွားနှုန်း အကောင်းဆုံးဖြစ်ကြောင်းလေ့လာတွေ့ရှိခဲ့ပါသည်။ Multiplication (ပွားများမွေးမြူခြင်း)စမ်းသပ်မှုတွင်တောင်ဇလပ်နီ ၏ ပွားများနှုန်းနှင့် ကြီးထွားနှုန်းကို သိရှိရန် အာဟာရမျိုးပွားစာ(Treatment) ၆ မျိုးဖြင့် သုံးကြိမ် စမ်းသပ်ခဲ့ရာတွင် Treatment 2 (Woody Plant medium (WPM) 3% sucrose, 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml)) သည် ပွားများနှုန်း နှင့် ကြီးထွားနှုန်း အကောင်းဆုံး ဖြစ်ကြောင်း တွေ့ရှိခဲ့ရပါသည်။

Study on growth performance of *Rhododendron arboreum* Sm.by seed culture

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Abstract

Rhododendron is a large genus of woody plants belongs to family Ericaceae and consists of around 1000 species within one genus and 8 sub-genera (Chamberlain et al., 1996). *Rhododendron arboreum* Sm. species naturally grow from seeds and regeneration rate is low. The investigation of seed germination is important for the conservation of this species. The sterilization experiment was carried out three kinds of sterilization methods (Sterilization A, B, C) with three times replicated and consisted of 40 seeds in one test tube. The lower contamination rate and high survival rate were the best sterilization in the sterilization B (Homine +Tween 20– 15 minute, Kasumin + Clorox -10 minute, HgCl2+Tween20 - 5 second, double distilled water (DDW) – 3 times) more than sterilization A and sterilization C. The induction experiment has six treatments with three times. The germination and growth percentage were the best treatment in Treatment 4 (Murashige and Skoog (MS) without hormone, 3% sucrose, 0.8% Agar) more effective than other treatments. The multiplication experiments also tested in six treatments with three times to know the multiple rate and growth rate of *Rhododendron arboreum* Sm. Among the Treatments, Treatment 2 (Woody Plant medium (WPM) 3% sucrose, 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml) was the most effective in growth media.

Keywords: ***Rhododendron arboreum*** **Sm., Sterilization method, Seed germination, Treatments**

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1. Introduction

Forests are important ecosystems because they provide countless ecosystem services. The name 'Rhododendron' is derived from the Greek word 'Rhodo' means rose and 'Dendron' means tree. Rhododendron is a large genus of woody plants belongs to family Ericaceae and consists of around 1000 species within one genus and 8 sub-genera (Chamberlain et al., 1996). Rhododendron have been recorded 205 species in Myanmar at checklist of Myanmar (Kress et al., 2003). It is extremely variable in stature, hardness, flower color and leaf characteristics. It grows at elevations of 4500 to 10,500 ft & grows up to 40 to 50 ft high sometimes attaining over 100 ft (Rai & Rai, 1994). This is an evergreen much branched tree up to 14 m in height & 2.4 m in girth (Chauhan, 1999). Flowering season is from March- April/ June-September bearing deep red or crimson to pale pink flowers. Most species are native to eastern Asia and the Himalayan region, but smaller numbers occur elsewhere in Asia, and in North America, Europe and Australia. The plant prefers light (sandy) to medium (loamy) soil & requires fairly moist & acidic soil. It can grow in semi shade (light woodland) or no shade, requires protection from hot afternoon sun thus requires a place in the green house or conservatory.

The Rhododendron is widely distributed in northern part of Myanmar and well known for its showy flowers and being representative flowers of Chin State (Kyi Kyi Khaing, 2015).In the Chin State, Myanmar, there are different climatic conditions according to the altitude and thus the forest structure changes with increase or decrease in elevation. It have various gorgeous, sacred, economic and medicinal values faces wide scale human interferences due to its over exploitation and unsustainable extraction making these species as nearly threatened (TinTinMu,2021). It is being usable as a ritual medicine for diverse diseases. Flowers, traditionally used by the people residing in the mountainous region to make pickle, juice, jam, syrup, honey, squash, etc. (TinTinMu,2021). One of the most important problems that mountainous forests are experiencing is poor regeneration (Karauchi et al. 2000). It is necessary to find alternative methods of propagation to ensure regular a supply of planting stock. Moreover, it is difficulty in multiplication, scanty information is available regarding seed culture methods of this species. Therefore, the present study was to explore on the growth performance of *Rhododendron arboreum* Sm. by seed culture method.

Botanical Characteristics of *Rhododendro arboreum* Sm.

Family	-	Ericaceae
Genus	-	Rhododendron
Species	-	*Rhododendron arboreum* Sm.
Myanmar Name	-	Taung Zalat Ni

The trunk is often much branched, crooked or gnarled (Orwa *et al.*, 2009). Bark is reddish brown, soft and rough, exfoliating in thin flakes (Chauhan, 1999). Leaves are oblong-lanceolate, 10-20 cm long and 3.6 cm wide. Crowded towards the ends of branches, petiole covered with white scales when young (Orwa *et al.*, 2009). It is glossy green, with deeply impressed veins from above white fawn, cinnamon or rusty brown felt is found at the under surface (Rai & Rai, 1994).

The flowers of *Rhododendron arboreum* range in color from a deep scarlet, to red with white markings, pink to white. Bearing up to twenty blossoms in a single truss this rhododendron is a spectacular sight when in full bloom. It is reported that the bright red

forms of this rhododendron are generally found at the lower elevations (Orwa *et al.*, 2009). Flowers are showy, red in dense globose cymes (Chauhan, 1999). Calyx- fine cleft, Corolla-tube spotted funnel shaped, Stamens-hypozygnous declining, Filaments- filiform, Anthers-ovate, Style-capitate (Paxton, 1834). Capsule-curved central column composed of fine lobes, ribbed, up to 3.8 cm long and 1.25 cm wide (Orwa *et al*., 2009). Seeds-minute, dark brown, compressed, thin linear having an obvolute membrane (Orwa *et al.*, 2009).

2. Objectives

The objectives of this research study are:

a) To explore the Sterilization methods
b) To find out the media formula for micro- propagation of induction stage
c) To find out the media formula for micro- propagation of multiplication stage

3. Literature Review

Rhododendron arboreum Sm. is a threatened species. Before disappearing this beautiful flowering plant from the earth, conservation and restoration programs are needed. Furthermore, Rhododendrons naturally grow from seeds, but their natural regeneration rate is low (Singh et al.,2008). The rate of vegetative propagation of many Rhododendrons is very slow (Mao et al.,2017). Although the capsules of Rhododendron produce hundreds of tiny seeds but seeds are too small to easily germinate in the soil because of low reserves of nutrients and the low number of viable seeds (Tiwari et al., 2007). The propagation from seeds is a prerequisite for establishing effective and efficient conservation practices (Shen et al.,2015).Seed plays a crucial role within the regeneration of tree species either by natural or artificial means(Bhatt et al.,2005). Seed germination is a key in the plant life cycle (Younesikelaki et al., 2016) and it directly affects plant reproductive success. The identification of seed germination requirements is more useful for its conservation programs. Many factors affect germination. The environment condition such as elevation effect and growing medium are important factors that can directly affect the success of seed germination.

Plant tissue culture is a perfect technique for plant genetic conservation. *In vitro* seed germination is the starting point and important stage for plant micro propagation (Ermayanti,et.al.,2014), and also it has been widely used on many plants to obtain seedlings from the seeds which are fail to germinate using conventional methods(lo,et.al.2018).

In vitro seed germination includes two main steps as sterilization protocol of seeds and selection of a suitable culture medium. The use of suitable sterilization method and culture medium leads to saving time, explants and effort (Amarasinghe, et.al, 2018).Also, it helps to overcome the difficulties encountered in (*in vitro*) propagation technique. Therefore, it is important to find out the suitable culture media and sterilization protocol for *in vitro* seed germination.

4. Materials and methods

4.1 Materials

4.1.1 Study Area

The study area is situated at the Natma Taung National Park (NTNP) in the southern Chin Hill of western Myanmar (between 20º45'-22º00' N and 93º15' -94º15' E), close to the border of India and Bangladesh. The Park covers 72.300 ha including three townships such as Mindat, Kanpetlet and Matupi and contains Mt. Natma Taung (Mt. Victoria), which rises 2.200 m above the surrounding landscape to a height of 3.051 m. It is the highest mountain in central Myanmar (Piatt *et al*. 2012).

The climatic of the Kanpetlet Township in the southern part of Chin State is characterized by its mild temperatures, high rainfall and humidity. From May to November are the rainy months, with this area getting more rain than other areas due to the monsoon storms come in from the Bay of Bengal. An annual rainfall is >1,000 mm; a cold dry season, maximum temperature varies between 4ºC (December- January) and a hot dry season with temperatures up to 20ºC (Fujikawa, 2009).

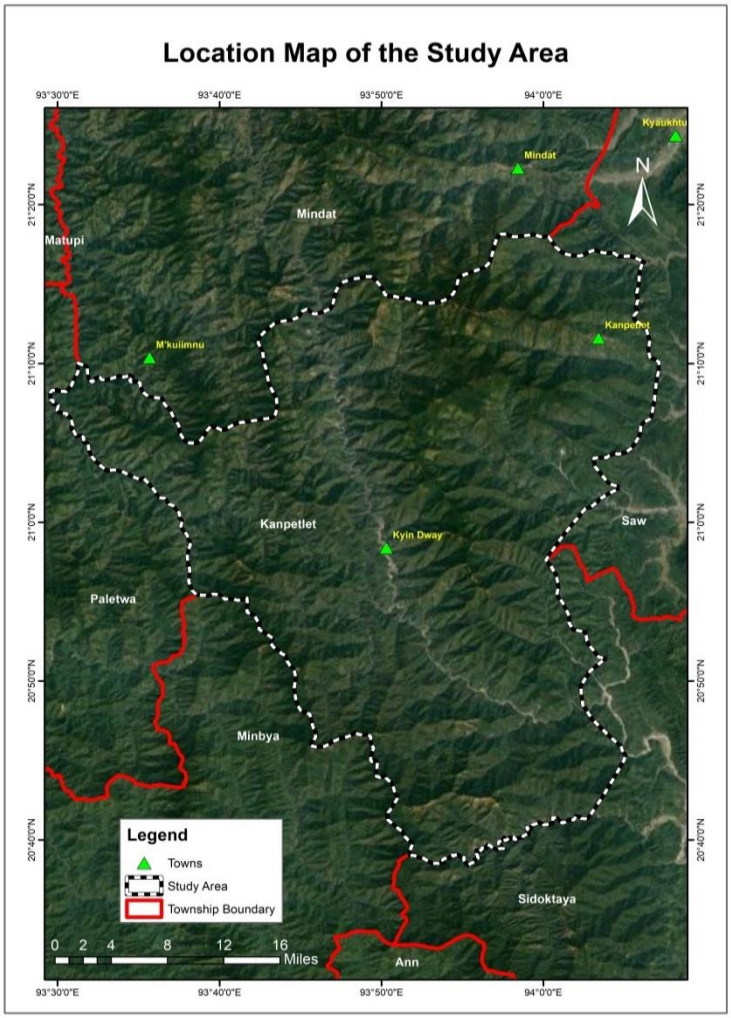


Figure 1. Location Map of the Study Area

4.1.2 Seed collection

Rhododendron arboreum fruits were collected in the month of October-December, 2019 from Natma Taung National Nature Reserve at an altitude of (N-21° 12' 20.6", E-94° 01' 24.43") and elevation at 2157 meter. When picking the fruit, cut it with secateurs, wrap it in tissue and collect it in a zip-lock bag. Collection date, collection place, number of fruits were recorded.

4.1.3 Sterilization of seed

The potential hurdle to getting a good supply of seeds for micro propagation is the risk of contamination from fungal or bacterial infection due to inefficient disinfection treatment. In testing the seeds with various pesticides, fungicide (Homine, Kasumin) Sodium hypochloride (Clorox) and Mercuric chloride ($HgCl_2$) were used for different durations to kill fungal or bacterial.

4.1.4 Culture media

Murashige and Skoog (MS 1962) medium and WPM (Lloyd and McCown, 1981) were used medium for seed tissue culture (*in vitro*). There were also tested for comparison of shoot induction and proliferation. The pH of the media was adjusted 5.4 to 5.7 with 1 N NaOH or HCl. Agar was added at 0.8 % w/v and melted in a microwave before dispensing into the culture vessels. Media were dispersed into borosilicate test tubes of the size 150 mm x 25 mm (20 ml media /tube) and 600 ml glass bottles with Plastic cap (40 ml/bottle). For sterilize, culture media were autoclaved at 1.05 kg/cm2 pressure at 12l˚C for 20 min.

4.1.5. Culture conditions

All cultures were maintained in the culture room where a temperature of 25° C ± 2 °C and 80% relative humidity. The photoperiod of 8 hr light (336.1 Lux)(fluorescent tubes- TL-D36W Philips, Snow White, Extreme Cool Daylight, made in PLAN) and 16 hrs darkness was provided to the cultures. Because this species take the time to grow in nature, so it takes 4-24 weeks in culture conditions after subculture onto fresh media. Depending on the growth hormone, the culture period was as long as 24 weeks.

4.2 Methods

There were three experiments in this study.

Experiment I. Effect of the best sterilization method on *Rhododendron arboreum* Sm. *in vitro* seed culture.

Experiment II. Effect of different media and growth rate on *Rhododendron arboreum* Sm. in induction stage *in vitro* seed culture

Experiment III. Effect of different media and growth rate on *Rhododendron arboreum* Sm. in multiplication stage *in vitro* seed culture

The fruit was collected from Nat Ma Taung National Park and taken to Forest Research Institute, Nay Pyi Taw. Rhododendron is a dehiscent fruit, so it can crack in the air. Rhododendron fruit were washed for 30 min in running tap water followed by detergent (Tween-20; 1.0%, v/v; 10 min) and in the shade dried. Rhododendron seeds are minute and many seeds. The obtained rhododendron seeds were purified and weighted in quantities for experiments.

4.2.1. Experiment I. Effect of the best sterilization method on *Rhododendron arboreum* Sm. in vitro seed culture.

In this experiment, completely randomized design was used and the contamination rate of seed sterilized with different sterilization methods, three times and three replicate within 16 weeks in tissue culture lab. One test tube consists of 40 seeds and one replicate consist of 60 test tubes. Measurement of this experiment is carried out weekly and the situation of contamination infection is carefully checked every day.

Sterilization name	Sterilization methods
Sterilization A	Homine(1g/1000ml) – 15 minute, Kasumin (5ml/100ml) – 10 minute, Clorox(5ml/100ml) -10 minute, $HgCl_2$ (0.02g/100ml) -3 second, double distilled water (DDW) – 3 times,
Sterilization B	Homine(1g/1000ml)+Tween 20(1-2 drop) – 15 minute, Kasumin (5ml/100ml) + Clorox (5ml/100ml) -10 minute, $HgCl_2$ (0.02g/ 100ml) + Tween20 (1-2 drop) - 5 second, double distilled water (DDW) – 3 times
Sterilization C	$HgCl_2$ (0.02g/100ml) + Tween 20 (1-2 drop) - 15minute, double distilled water (DDW) – 3 times.

4.2.2. Experiment II. Effect of different media and growth rate on *Rhododendron arboreum* Sm in induction stage *in vitro* seed culture

In this experiment, completely randomized design, six treatments for induction stage with three replication. In this experiment to germinate proliferation *in vitro*, following culture media were tested:

Treatment name	Treatment methods
Treatment 1	Woody Plant medium (WPM) without hormone ,3% sucrose , 0.8% Agar
Treatment 2	Woody Plant medium (WPM), 3% sucrose, 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml)
Treatment 3	Woody Plant medium (WPM), 3% sucrose, 0.8% Agar supplemented with BAP (10ml), NAA (10ml)
Treatment 4	Murashige and Skoog (MS) without hormone ,3% sucrose, 0.8% Agar
Treatment 5	Murashige and Skoog (MS),3% sucrose, 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml)
Treatment 6	Murashige and Skoog (MS),3% sucrose, 0.8% Agar supplemented with BAP (10ml), NAA (10ml)

4.2.3. Experiment III. Effect of different media and growth rate on *Rhododendron arboreum* Sm. in multiplication stage *in vitro* seed culture

In this experiment, completely randomized design, six treatments for induction stage with three times replicated. In this experiment to germinate proliferation *in vitro*, following culture media were tested:

Treatment name	Treatment methods
Treatment 1	Woody Plant medium (WPM) without hormone ,3% sucrose , 0.8% Agar
Treatment 2	Woody Plant medium (WPM) 3% sucrose , 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml)
Treatment 3	Woody Plant medium (WPM) ,3% sucrose , 0.8% Agar supplemented with BAP (10ml), NAA(10ml)
Treatment 4	Murashige and Skoog (MS) without hormone ,3% sucrose , 0.8% Agar
Treatment 5	Murashige and Skoog (MS),3% sucrose , 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml)
Treatment 6	Murashige and Skoog (MS),3% sucrose , 0.8% Agar supplemented with BAP (10ml), NAA(10ml)

4.2.4. Statistical analysis

All results were subjected to analysis of variance (ANOVA) with F tests between means and evaluated significant treatment effects at a significance level of $p \leq 0.05$ by using SPSS 26 statistical software. The standard deviation (SD) and the least significant difference (LSD) were calculated to compare the differences between means in each treatment. Excel and Microsoft Office (2010) were used for the assessment of the significance of the data.

5. Results and Discussions

5.1 Experiment I. Effect of the best sterilization method on *Rhododendron arboreum* Sm. *in vitro* seed culture

It was observed that Sterilization A and C are initiates contamination within 3rd weeks. During 16 weeks, Sterilization B is contaminating infection. Sterilization B is the effective method more than Sterilization A and Sterilization C. Sterilization B was the lowest contaminate (21.11%), followed by the Sterilization A (46.11%) and Sterilization C(37.77%) was significantly different ($p<0.05$) (Table 1, Fig 2) . The contamination rate was still higher when Sterilization A and Sterilization C. Sterilization A and B are significant different but Sterilization A and C are not significant different.

The result that the survival rate of seed sterilized with different sterilization methods, Sterilization B is the most significantly high among three different sterilization methods. Sterilization B was the highest survival (78.89%), followed by the Sterilization A (53.89%) and Sterilization C (62.22%) was significantly different ($p<0.05$) . The survival rate was still lower when Sterilization A and Sterilization C. Sterilization A and B are significant different but Sterilization A and C are not significant different. Therefore, the sterilization B had the highest survival rate (78.89%) (Table 1, Fig 3) due to a lower contamination rate.

Table1. Effect of sterilization method on contamination and survival rate of *Rhododendron arboreum.* Sm. Mean value ± Standard Deviation is showed. Different letters within a row ndicate a significant difference between sterilization methods ($p<0.05$, LSD test).

Methods	Contamination (%)	Survival (%)
Sterilization A	47.67 ± 6.94 [b]	52.33 ± 6.94 [b]
Sterilization B	21.11± 9.18 [a]	78.89 ± 9.18 [a]
Sterilization C	37.77 ±11.83 [b]	62.22 ± 11.82 [b]

Note; Sterilization A (Homine – 15 minute, Kasumin – 10 minute, Clorox -10 minute, $HgCl_2$ -3 second, double distilled water (DDW) – 3 times), Sterilization B (Homine +Tween 20– 15 minute, Kasumin + Clorox -10 minute, $HgCl_2$+Tween20 - 5 second, double distilled water (DDW) – 3 times), Sterilization C ($HgCl_2$+Tween 20 - 15minute, double distilled water (DDW) – 3 times).

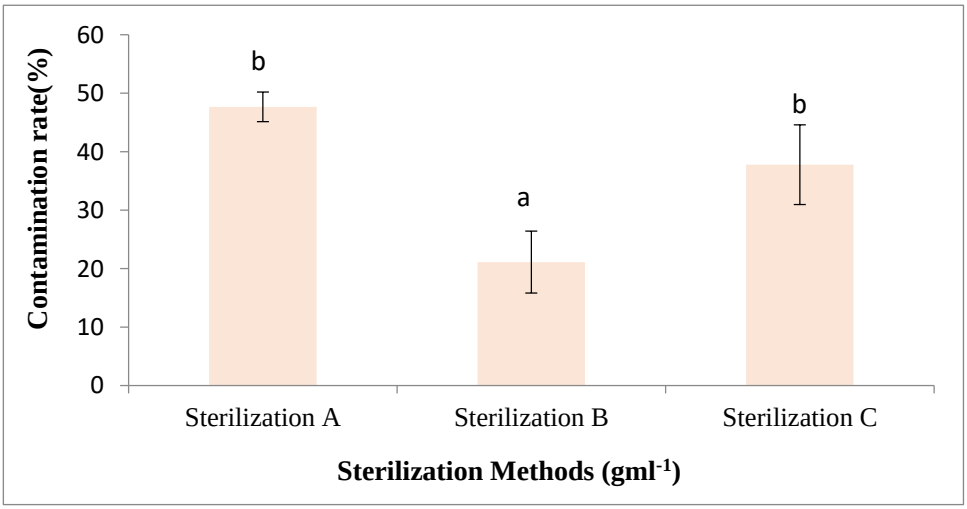


Figure 2. *R. arboreum* effect of contamination by Sterilization methods

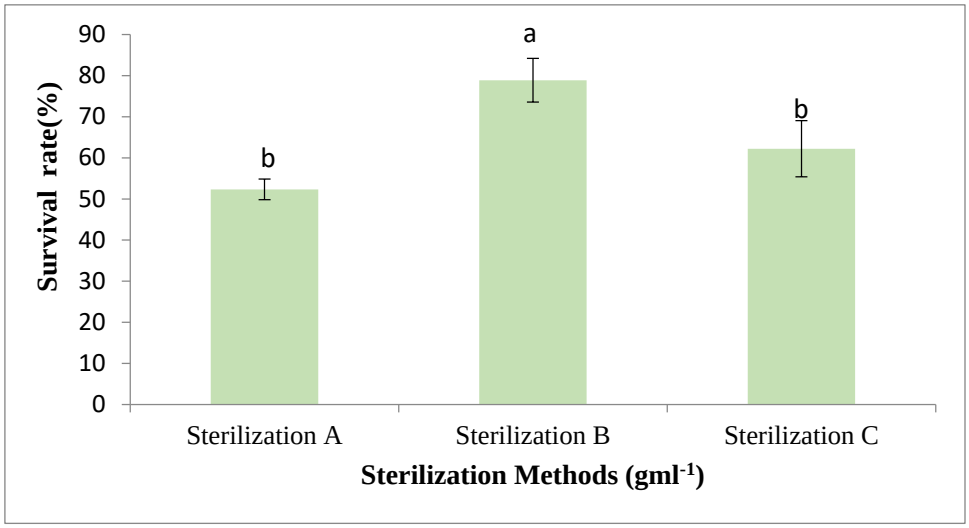


Figure 3. *R. arboreum* effect of survival by Sterilization methods

Figure 4. *Rhododendron arboreum* Sm. fruits

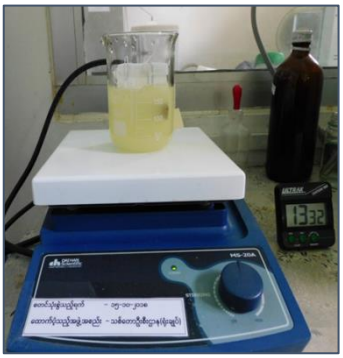


Figure 5. Sterilization with fungicide

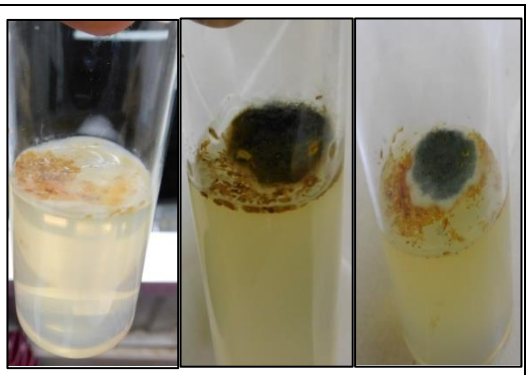


Figure 6. Contamination (fungius and bacteria)

(Amarasinghe, et.al, 2018) has also found that seeds could be sterilized with 15% NaOCl + Tween 20 for 20 min produced 100% contamination-free cultures. (Ermayanti., 2014) has also observed that seeds were surface-sterilized using Sodium hypochloride NaOCl. (S.H. Sandeepani1, 2021) has also observed that seeds could be sterilized with 20% Clorox with few drops of Teepol (detergent) for 10 minutes. In this experiment, NaOCl Sodium hypochloride, mercuric chloride $HgCl_2$ and Tween 20 were used with supplemented fungicide (Homine, Kasumin). So, these experiment results are nearly similar with my experiment.

5.2. Experiment II. Effect of different media and growth rate on *Rhododendron arboreum* Sm in induction stage *in vitro* seed culture

In this experiment, seed germination of *Rhododendron arboreum* Sm. (*in vitro*) with six treatments (different media), three times and three replicate in induction stage. This experiment was measure of weekly out and the germination of seed is carefully checked out within 24 weeks. Treatment 4 is the most effectively high among six treatments as it is the high germination of seed by the statistical analysis. Treatment 4 is the highest germination - 23.54 ($p< 0.05$), followed by Treatment 1- 13.62($p< 0.05$), Treatment 2- 4.90($p< 0.05$), Treatment 3-3.78($p< 0.05$), Treatment 5-10.60 ($p< 0.05$) and Treatment 6- 8.73($p< 0.05$). T2

and T3 are not significant different other treatment and T1, T4, T5 and T6 are significant different. So, T4 is the best treatment germination (Table 2, Fig.7) and T2, T3 are lowest germination treatments.

In this experiment, growth of *Rhododendron arboreum* Sm. with six treatments (different media), three times and three replicate in induction stage. Measurement of this experiment is length (cm) for shoot growth within 24 weeks. Treatment 4 is the most significantly different treatment among six treatments as it is the high growth of shoot. In the result, Treatment 4 is the highest growth -12.51 ($p< 0.05$) , followed by T2-2.80 ($p<0.05$) , T5- 5.75 ($p<0.05$) ,T6- 4.75 ($p<0.05$), T1-7.26($p<0.05$) and T3-2.08 ($p<0.05$) are respectively. In there, T5 and T6 are not significant different but other treatment 1, 2, 3 and 4 with significant difference and also same that T2 and T3 (Table-2, Fig.8).

Table2. Effect of germination and growth rate of *Rhododendron arboreum*. Sm. Mean value ± Standard Deviation is showed. Different letters within a row indicate a significant difference between six treatments of media in induction stage ($p<0.05$, LSD test).

Treatments	**Number of Germination**	**Length of shoot growth(cm)**
Treatment 1	13.62 ± 2.97 ^{b}	7.26 ± 2.06 ^{b}
Treatment 2	4.90 ± 2.11 ^{e}	2.80 ± 1.77 ^{d}
Treatment 3	3.78 ± 2.12 ^{e}	2.08 ± 7.09 ^{d}
Treatment 4	23.54 ± 3.83 ^{a}	12.51 ± 2.27 ^{a}
Treatment 5	10.60 ± 4.11 ^{c}	5.75 ± 2.16 ^{c}
Treatment 6	8.73 ± 4,51 ^{d}	4.75 ± 2.43 ^{c}

Note; Treatment 1: WPM without hormone ,3% sucrose , 0.8% Agar and Treatment 2: (WPM), 3% sucrose , 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml), and Treatment 3: (WPM) ,3% sucrose , 0.8% Agar supplemented with BAP (10ml), NAA(10ml), Treatment 4: (MS) without hormone ,3% sucrose , 0.8% Agar and Treatment 5: (MS),3% sucrose , 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml), and Treatment 6: (MS),3% sucrose , 0.8% Agar supplemented with BAP (10ml), NAA(10ml)

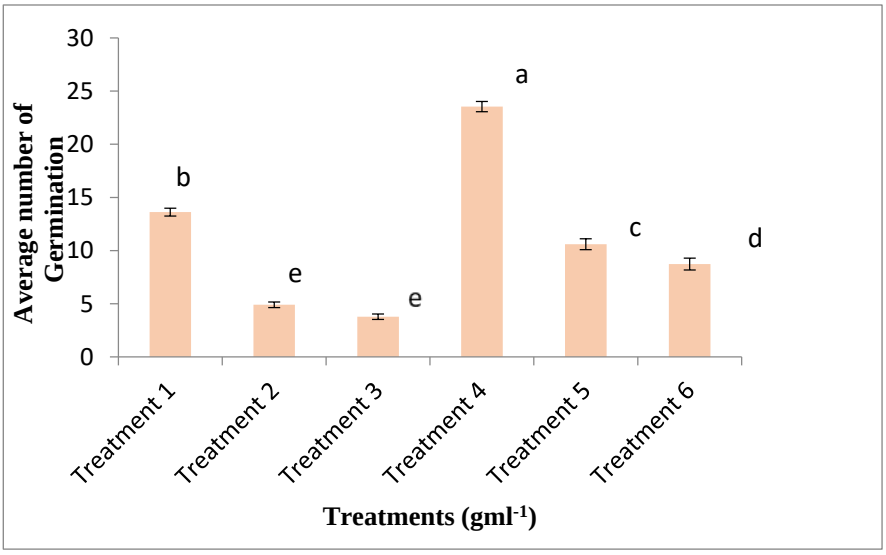


Figure 7. *R. arboreum* effect of germination in treatments

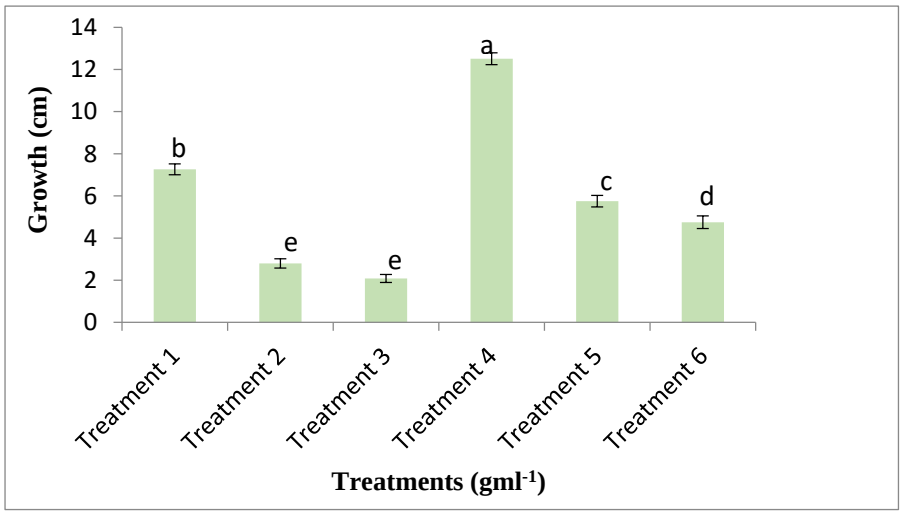


Figure 8. *R. arboreum* effect of growth in treatments

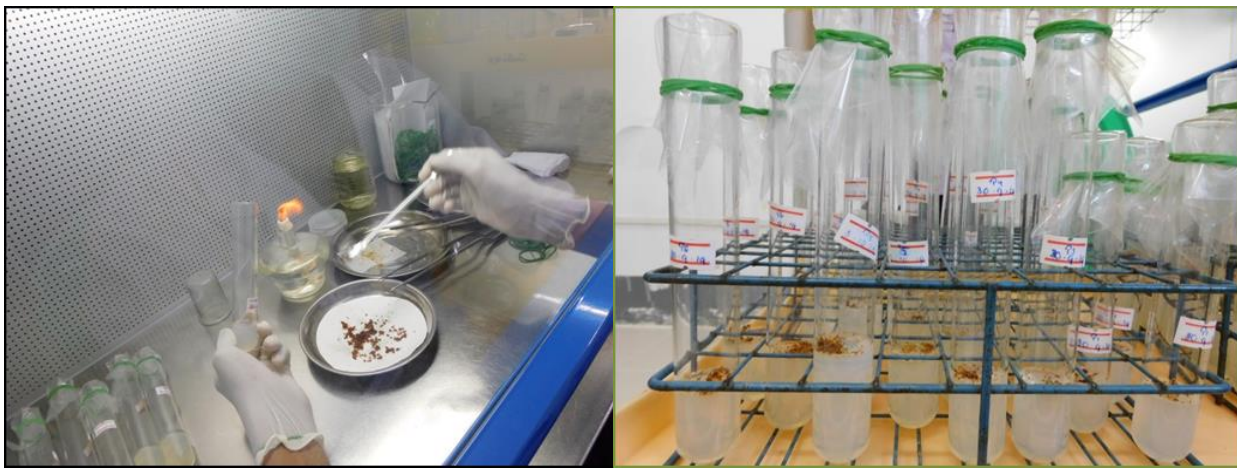


Figure 9. Six kinds of Treatments experiment in induction stage of *R. arboreum* Sm.seeds

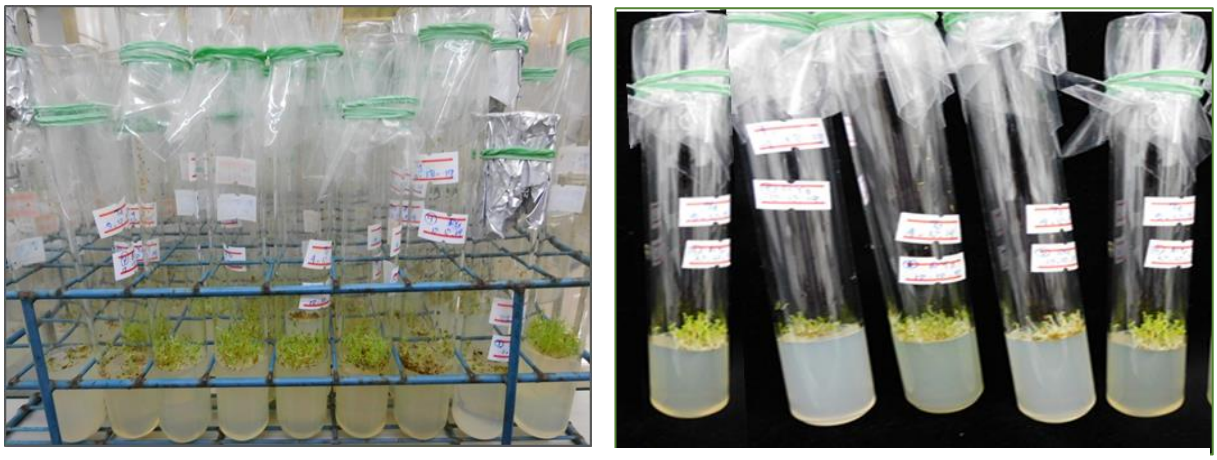


Figure 10. Germination and growth rate of six treatments in induction stage of *R. arboreum* Sm. seeds

(Kumar et al., 2004) has read that full MS medium(without hormone) was effective for seed germination of *R. maddeni* at the initial stage and (Ermayanti.,2014) has also observed that seed of *Rhododendron correoides and Rhododendron culminicolum* germinated aseptically on Murashige and Skoog (MS) medium(free hormone) at the initial stage. These

experimental results much the same with my found of experiment. (Weerasinghe P.A,2021) has regarded that *Rhododendron arboreum* subsp. *zeplanicum* ,In the (*in vitro*) seed germination, the lowest germination were recorded in the full-strength MS medium(without hormone). Therefore, these experimental results similarly with this present study.

5.3. Experiment III. Effect of different media and growth rate on *Rhododendron arboreum* Sm. in multiplication stage *in vitro* seed culture

In this experiment, multiple of *Rhododendron arboreum* Sm. (*in vitro*) with six treatments (different media), three times and three replicate in multiplication stage. This experiment was measure of weekly out and the multiple of shoot is carefully checked out within 24 weeks. In this result, Treatment 3 is callus formation (brown) without multiplicative during 12 weeks (Fig-15) and Treatment 6 resulted in plant death without multiplicative (Fig-14). In among six treatments, Treatment 2- 22.63 ($p<0.05$) is plenty of seedling in multiplication stage than Treatment 1- 5.20 ($p<0.05$), Treatment 4- 14.15 ($p<0.05$), Treatment 5- 5.20 ($p<0.05$) respectively. T3 and T6 are not significant different other treatment and T1, T4, T5 are significant different. So, T2 is the most effective treatment multiple rate in this experiment. (Table3, Fig 11)

In this experiment of multiplication stage, Treatment 3 resulted callus formation without growth and treatment 6 resulted plant death without growth height of *Rhododendron arboreum* Sm. In among six treatments, Treatment 2- 19.94 ($p<0.05$) is growth height of seedling in multiplication stage than Treatment 1- 6.25($p<0.05$), Treatment 4- 11.89 ($p<0.05$), Treatment 5- 5.18 ($p<0.05$) respectively.T3 and T6 are not significant different other treatment and T1, T4, T5 are significant different. So, T2 is the best treatment growth height in this experiment. (Table3, Fig 12)

Table3. Effect of multiplicate and growth rate of *Rhododendron arboreum*. Sm. Mean value ± Standard Deviation is showed. Different letters within a row indicate a significant difference between six treatments of media in multiplication stage ($p<0.05$, LSD test).

Treatments	Mean number of Multiplication rate	Growth rate (cm)
Treatment 1	5.33 ± 0.47^{c}	6.25 ± 0.95^{d}
Treatment 2	22.63 ± 1.88^{a}	19.94 ± 1.58^{a}
Treatment 3	0.99 ± 0^{d}	0.99 ± 0^{e}
Treatment 4	14.15 ± 2.77^{b}	11.89 ± 2.61^{b}
Treatment 5	5.20 ± 0.401^{c}	5.18 ± 0.93^{c}
Treatment 6	0.99 ± 0^{d}	0.99 ± 0^{e}

Note: Treatment 1: WPM without hormone ,3% sucrose , 0.8% Agar and Treatment 2: (WPM), 3% sucrose , 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml), and Treatment 3: (WPM) ,3% sucrose , 0.8% Agar supplemented with BAP (10ml), NAA(10ml), Treatment 4: (MS) without hormone ,3% sucrose , 0.8% Agar and Treatment 5: (MS),3% sucrose , 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml), and Treatment 6: (MS),3% sucrose , 0.8% Agar supplemented with BAP (10ml), NAA(10ml)

The full MS medium the type and concentration of the cytokinin had a significant effect on the frequency of shoot regeneration, shoot number and shoot length as also seen in (Kumar et al., 2004). These experimental result also not similarly with my found of experiment. *R. culminicolum* callus was better when they were cultured on WP medium compared to MS medium. In addition, the formation of multiple shoots was faster and higher on WP medium than that on MS medium in (Ermayanti, M. T., 2014). These observed result also same that in my experiment.(Sicuranza. J., Mitkowski. N. A., 2007) examined that for callus and shoots using Anderson's medium , IAA and 2ip in the *Rhododendron catawbiense* (Michx.) varieties Roseum Elegans and English Roseum. (Qazi.A.I., 2021) callus production was observed on Woody Plant Medium supplemented with 2,4-D (1mg/l) within 35 days of culture with a percent response of 70% from Rhododendron of the seeds was inoculated. In this experiment, used WPM medium, BAP and NAA treatment was found that callus formation.

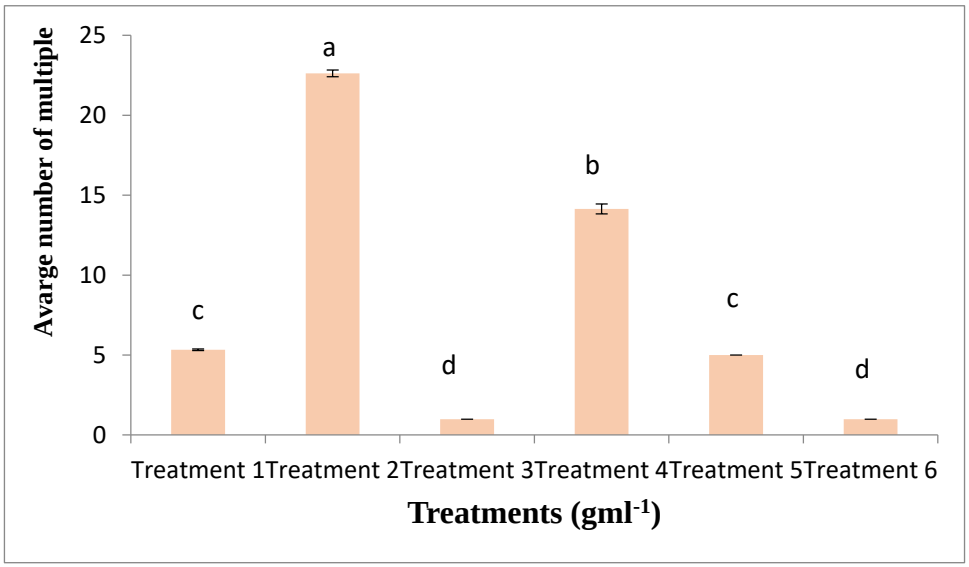


Figure 11. *R. arboreum* effect of treatments on multiplication

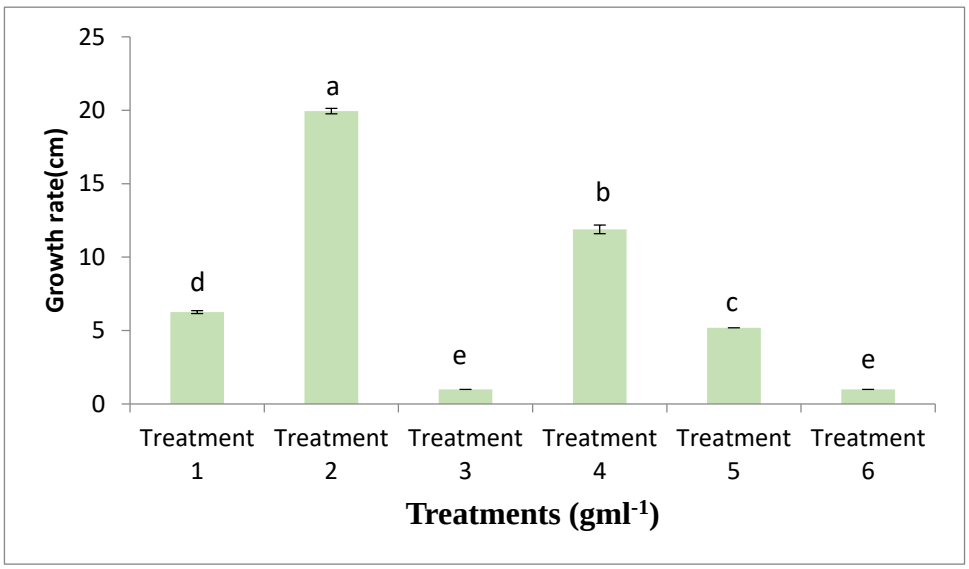
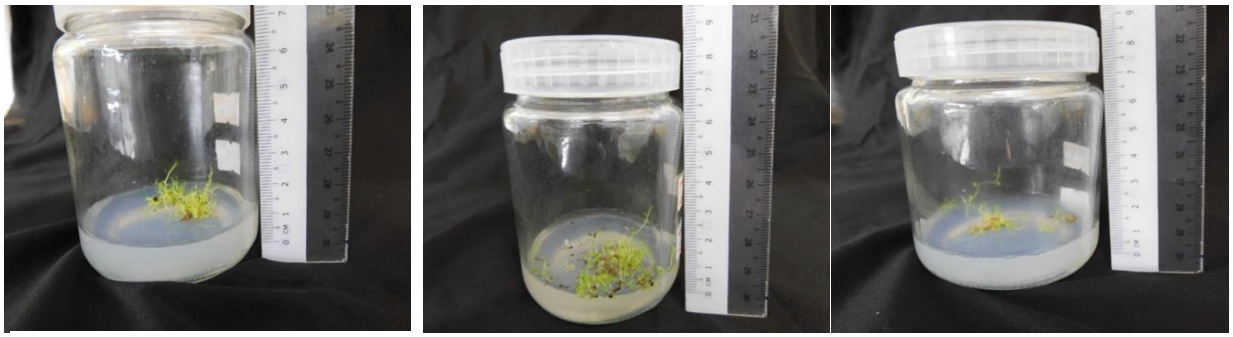
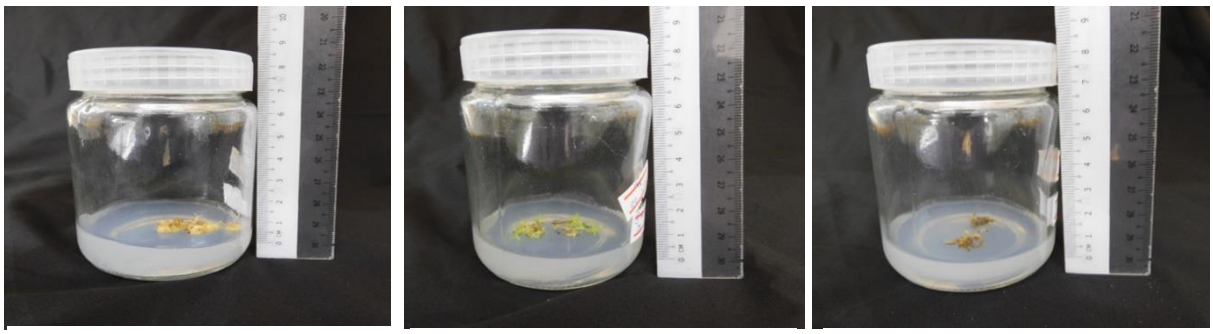


Figure 12. *R. arboreum* effect of treatments on growth



T2 T4 T1

Figure 13. Multiple and growth rate of six treatments in multiplication stage of good culture *R. arboreum* Sm.



T3 T5 T6

Figure 14. Multiple and growth rate of six treatments in multiplication stage of bad culture *R. arboreum* Sm.

6. Conclusion

Based on the result of the present study, the following conclusion can be drawn; seeds of *Rhododendron arboreum* Sm. explored with different sterilization process. Sterilization B is the good performance. Sterilization B is contaminating infection during 16 weeks. Sterilization B was the lowest contaminate (21.11%) and highest survival rate (78.89%) than Sterilization A and C. Sterilization B is the effective method more than Sterilization A and Sterilization C. The plantlets get the less disinfection due to exploring the effective method of sterilization in (*in vitro*) seed culture.

At the induction stage, among the six treatments, Treatment 4 is the most effective in germination and growth rate of *Rhododendron arboreum* Sm. seeds. Treatment 4 is the highest germination -23.54 and the highest growth -12.51 than other treatments. Treatment 4 is without hormone. So it use can be low cost for shoot hormone cost.

Moreover, in multiplication stage, Treatment 2 is an effective result of *Rhododendron arboreum* Sm. seeds. Treatment 2 is average of multiple rate (22.63) and growth rate (19.94cm) other than Treatment 1,3,4,5 and 6. Treatment 2 is (WPM), 3% sucrose, 0.8% Agar supplemented with 2iP (6ml), Zeatin (3ml), so it media is multiplication, growth of shoot and lead to saving time.

7. Recommendation

Rhododendron arboreum Sm. is a threatened species. Before disappearing this beautiful flowering plant from the earth, conservation and restoration programs are needed. *Rhododendron arboreum* Sm. is a slow growing species in nature, the micro propagation method makes it grow a little faster than nature. In this research, *Rhododendron arboreum* Sm. used (*in vitro*) seed culture exploring the effective method of sterilization, found out the most effective of media for seed germination and survival, and found out the best of media for multiplication and growth. The plants obtained from propagation should be replanted in the original area after rooting.

8. Acknowledgements

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Appendix I

Murashige and Skoog's -1962 (MS) and Woody Plant Medium - 1981(WPM)

Chemical Components	**(MS) Concentration in medium ($mg.L^{-1}$)**	**(WPM) Concentration in medium ($mg.L^{-1}$)**
NH_4NO_3	1.65	400.00
KNO_3	1.90	-
$CaC1_2.2H_2O$	0.44	96.00
$MgSO_4.7H_2O$	0.37	370.00
KH_2PO_4	0.17	170.00
$FeSO_4.7H_2O$	27.8	27.80
Na_2 EDTA	37.3	37.30
H_3BO_3	6.2	6.20
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.4H_2O$	8.6	8.6
KI	0.83	-
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25
$CoCl_2.6H_2O$	0.025	-
Nicotinic acid	0.5	0.5
Pyrioxidine. HCl	0.5	0.5
Thiamine. HCl	0.1	0.1
Glycine	2	2
Myoinositol	0.1	0.1

Appendix II

Table 1. Tests of Sterilization Methods Effects ANOVA					
Dependent Variable: Contamination					
Source	Sum of Squares	df	Mean Square	F	Sig.
Between groups	1080.558	2	540.279	6.665	.030
Within groups	486.363	6	81.061		
Total	1566.921	8			

The mean different is significant at the 0.05 level.
(*R. arboreum* for Contamination)

Table 1. Tests of Sterilization Methods Effects ANOVA					
Dependent Variable: Survival					
Source	Sum of Squares	df	Mean Square	F	Sig.
Between groups	1080.558	2	540.279	6.665	.030
Within groups	486.363	6			
Total	1566.921	8			

The mean different is significant at the 0.05 level.
(*R. arboreum* for Survival)

Table 2. Tests of Treatments Effects ANOVA					
Dependent Variable: Germination					
Source	Sum of Squares	df	Mean Square	F	Sig.
Between groups	16292.529	5	3258.506	280.054	.000
Within groups	4328.317	372	11.635		
Total	20620.847	377			

The mean different is significant at the 0.05 level.
(*R. arboreum* for Germination)

Table 2. Tests of Treatments Effects ANOVA					
Dependent Variable: Growth					
Source	Sum of Squares	df	Mean Square	F	Sig.
Between groups	4479.157	5	895.831	210.449	.000
Within groups	1583.519	372	4.257		
Total	6062.676	377			

The mean different is significant at the 0.05 level.
(*R. arboreum* for Growth)

Table 3. Tests of Treatments Effects ANOVA					
Dependent Variable: Multiplication					
Source	Sum of Squares	df	Mean Square	F	Sig.
Between groups	29647.513	5	5929.503	3103.399	.000
Within groups	917.111	480	1.911		
Total	30564.624	485			

The mean different is significant at the 0.05 level.
(*R. arboreum* for Multiplication)

Table 3. Tests of Treatments Effects ANOVA					
Dependent Variable: Growth					
Source	Sum of Squares	df	Mean Square	F	Sig.
Between groups	21531.456	5	4306.291	2322.796	.000
Within groups	889.884	480	1.854		
Total	22421.340				

The mean different is significant at the 0.05 level.
(*R. arboreum* for growth)