Pollen storage, viability and effect of growth hormones on in vitro pollen germination in two medicinal plants (Clerodendrum colebrookianum Walp. and Clerodendrum infortunatum L.) of the tropical moist forest of North-east India

Puyam Devanda Singh  
Department of Forestry, Mizoram University, Aizawl -796004 (Mizoram), India

Kewat Sanjay Kumar*  
Department of Forestry, Mizoram University, Aizawl -796004 (Mizoram), India

Vinod Prasad Khanduri  
College of Forestry, VCSG Uttarakhand University of Horticulture and Forestry, Ranichauri, Tehri Garhwal- 249199 (Uttarakhand), India

*Corresponding author email: kewatsanjay@gmail.com

How to Cite

Abstract
Clerodendrum colebrookianum Walp. and Clerodendrum infortunatum L. are well known for their medicinal uses in treating various human ailments traditionally. Pollen biology study was done in both plant species to decipher pollen viability, in vitro pollen germination, and pollen storage in varied temperature conditions. Pollen viability tests was done by 2, 3, 5-triphenyl tetrazolium chloride (TTC test) for which pollen grains were collected at the anthesis stage that ascertained high viability, ranged between 71.97%±4.30 % in C. colebrookianum and 81.63%±3.23 in C. infortunatum. In vitro pollen germination was conducted under different hormones (IBA, IAA, GA3, and Kinetin) with concentrations of 100 mg L⁻¹, 200 mg L⁻¹ and 300 mg L⁻¹. Highest germination percentage of 52.10±5.30% and 61.91±1.76% in GA3 (200 mg L⁻¹) was the most suitable growth hormone concentration for inducing in vitro pollen germination in both Clerodendrum species. Statistically, the response of all the concentrations of treatments, sucrose, and hormones, with their time on in vitro pollen germination of C. colebrookianum and C. infortunatum was found significantly different (p<0.05). In contrast, non-significant differences were recorded for in vitro pollen germination between the medicinal plant species. Pollen storage under temperature gradient conditions exhibited a similar trend in the viability for both C. colebrookianum, and C. infortunatum, i.e., the pollen remained viable up to 28 days at -20°C and 6°C, respectively. This study will be helpful in future breeding, hybridization, and conservation efforts for both the Clerodendrum species.

Keywords: Clerodendrum colebrookianum, Hormones, Medicinal uses, Pollen germination

INTRODUCTION

Genus Clerodendrum (Family: Lamiaceae) is a well-known genus for its medicinal uses in indigenous systems of medicines for treating various diseases across cultural landscapes. C. colebrookianum and C. infortunatum L. are valuable and popular medicinal plant resources in the genus. C. colebrookianum is a vulnerable flowering shrub (Gogoi and Nath, 2021). C. colebrookianum is a medicinal food plant, and its leaves are used as a vegetable. Local people of the Northeast region of India commonly use the leaf vegetable of C. colebrookianum as a home remedy for high blood pressure. It is highly valued in the treatment of natural anti-hypertension, antidiabetic, hepatoprotective, and sedative properties (Das et al., 2015 and Arya et al., 2018). The traditional practitioners in Mizoram commonly used its leaves as a cardioprotective against diarrhoea and dysentery and as anti-colic for infants (Lokesh and Amitsankar, 2012). Local inhabitants of Assam, Arunachal Pradesh, Manipur, and Nagaland used leaves extract and its decoction for abdominal pain, dizziness, gastric disorders, dysentery treatment, cough, skin diseases, and anthelmintic (Kalita et al., 2012; Murtem...
Shivanna, 2016; Jamir and Tsurho, 2016; Yadav et al., 2018 and Kshetri et al., 2022). Leaves were reported to possess clerosterol, colebrin, sitosterol, octacosanol, daucosterol, and fatty acids (Yang et al., 2000). C. infortunatum is a shrub medicinally useful in relieving thirst and burning sensation, foul orders, and blood diseases (Rej et al., 2014). Leaf extracts are effective against scorpion sting, pain reliever, and act as expectorant and vermifuge, while bark juice relieves abdominal pain and indigestion (Nandi and Lyndem, 2016). In homeopathy, it is used as remedial medicine for diarrhoea and fresh wounds (Helen et al., 2021). C. infortunatum is reported to have several pharmacological properties, viz. anthelmintic, anticonvulsant, analgesic, wound healing, antioxidant, anticancer, antimalaria, and antifungal activities (Bhattacharjee et al., 2011 and Saha et al., 2018). C. infortunatum also contains sapo- nin, diterpene (Clerodin), triterpene (luepol), steroid (β-sitosterol), flavonoids, glycerides of stearic acid, linoleic acid, and lignoceric acid (Bhattacharjee et al., 2011). Compared to the medicinal importance of C. colebrooki- anum and C. infortunatum, there are minimal studies on its propagation (Mao et al., 1995) and reproductive biology. Pollen viability, germination, and storage are es- tential aspects of reproductive biology and breeding of a plant species, as viable and fertile pollen is critical for efficient sexual plant reproduction. The pollen’s viability and vigor determine the pollen quality rate, which is crucial in artificial pollination and inbreeding experiments for understanding sterility and hybridization (Shivanna, 2019). Proper germination and growth of pollen grains are essential for fertilization, fruit, and seed development (Shivanna and Rangaswamy, 2012). in vitro pollen germination is used significantly on a vari- ety of pollen frameworks (Hao et al., 2022). The study of pollen germination is vital in plant developmental biology. It can provide abundant knowledge on the nutri- tional and physiological requirements of pollen germi- nation and its growth (Shivanna and Rangswamy, 2012). A linear relationship between pollen viability and pollen germination was observed, and in numerous plant species, it has got direct correlation with fruit and seed set (Abdelgadir et al., 2012; Mesnoua et al., 2018 and Shivanna, 2019). The storage of viable pollen under controlled conditions is valuable in breeding programs, genetic preservation, artificial fertilization, and self-incompatibility (Shivanna, 2019). The life span of pollen varies significantly with plant species and storage conditions (Mesnoua et al., 2018). Organic solvents, refrigeration, freeze-drying, and cryopreservation are distinctive strategies for pollen storage (Sidhu, 2019). The duration of viable pollen storage can be expanded by regulating temperature, relative humidity, and storage atmosphere (Mesnoua et al., 2018; Jaskani and Naqvi, 2017).

Looking into the economic medicinal importance of C. colebrookianum and C. infortunatum, the present study was conducted to discern the influence of growth regulators and time on in vitro pollen germination and pollen storage for its application in future breeding and con- servation program.

MATERIALS AND METHODS

Two species of Clerodendrum, viz. C. colebrookianum and C. infortunatum (Family: Lamiaceae) were selected at Tanhril village (23° 44ʹ 15ʺ N, 92° 39ʹ 44ʺ E, 748 m asl) in the district Aizawl, Mizoram. The study was con- ducted during the year 2019. In C. colebrookianum, flowering occurred between July to December (2019), while in C. infortunatum, it occurred between February to April, 2019 at the forest site. Freshly opened flower samples at the anthesis stage were collected in the morning (6-9 am) from five individuals growing 100 m apart from each other from the forest site for experiments. Unopened flowers (just prior to anthesis) and just opened flowers were chosen to check the pollen viability. 0.5 percent 2, 3, 5-triphenyl tetrazolium chloride (TTC) prepared in the sucrose so- lution was used to check the pollen viability. A small number of pollen grains were suspended in the TTC (0.5%) solution and were covered with cover galas. Slides were incubated in darks for 60 minutes. After the incubation period, the preparation was observed under a light microscope (5 X & 10 X); pollen grains stained red are counted as viable (Shivanna and Rangaswamy, 2012).

Brewbaker and Kwack’s (1963) basal medium and method was used for in vitro pollen germination stud- ies. 5% and 10% sucrose concentrations were used to analyze the effect of sucrose against control (distilled water). Growth regulators, viz., Indole-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), Gibberellic Acid (GA₃), and Kinetin and their concentrations of 100, 200, and 300 ppm were supplemented in the basal medium to check their effects on in vitro pollen germination. A randomized complete block design with five replications blocked in time was used in the experiment (Tuinstra and Wedel, 2000). With the help of a needle, pollen grains from fresh anthers were put on germination me- dia in the cavity slides. The cavity slides were placed in room conditions; average temperature (26.35±0.98) and average humidity (79.58±3.07) were recorded with a thermo-hygrometer. After incubation intervals of 24, 48, and 72 hours cavity slides were observed under a light microscope. Pollen grains were considered to be germinated when pollen tube length was found to be greater or equal to the diameter of pollen grains (Tuinstra and Wedel, 2000). The pollen grains in each germination cavity slide were assessed in 10 micro-
scopie views; a total number of germinated and non-germinated pollen grains were counted in each view and expressed as a percentage of in vitro pollen germination. Statistical technique ANOVA was used to analyze the effect of hormones and their concentrations, sucrose concentrations, time, and plant species were assessed for in vitro pollen germination with the help of Excel 2016.

Fresh pollen grains were stored under an air-tight vial at three different temperatures, i.e., 6°C, -4°C, and -20°C. The viability of stored pollen grains was tested regularly with 0.5% TTC at an interval of 24 hours for seven days after that; viability was checked at an interval of a week, i.e., 14, 21, and 28 days, respectively, under a light microscope until pollen grains were found to be viable.

RESULTS

Pollen grains viability percentage in pre-anthesis stage (un-opened flower) was 28.57% ± 2.61% and in freshly opened flower (at anthesis stage) was 71.97% ± 4.30% in case of C. colebrookianum while in case of C. infortunatum it was 19.37% ± 1.73% in pre-anthesis stage and 81.63%±3.23 during anthesis stage. Hence anthesis stage pollen grains were found to be more viable and suitable for pollination in both plant species (Table 3).

In vitro pollen grain germination showed a differential response with varied growth regulators (IAA, IBA, GA3, and Kinetin), sucrose concentrations, and time in both the Clerodendrum species. In control (distilled water), exceptionally very low percentage of pollen germination was observed. Table 1. Effect of growth hormones on in vitro pollen germination in two Clerodendrum species

Table 1. Effect of growth hormones on in vitro pollen germination in two Clerodendrum species

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Concentrations</th>
<th>C. colebrookianum</th>
<th>C. infortunatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
<td>48 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>IAA (10% Sucrose)</td>
<td>100 mg L⁻¹</td>
<td>39.88±3.70</td>
<td>7.12±0.81</td>
</tr>
<tr>
<td>IBA (10% Sucrose)</td>
<td>200 mg L⁻¹</td>
<td>31.80±1.97</td>
<td>4.73±0.61</td>
</tr>
<tr>
<td>IBA (10% Sucrose)</td>
<td>300 mg L⁻¹</td>
<td>26.86±3.08</td>
<td>4.64±0.70</td>
</tr>
<tr>
<td>IBA (10% Sucrose)</td>
<td>100 mg L⁻¹</td>
<td>46.56±4.59</td>
<td>9.20±0.90</td>
</tr>
<tr>
<td>IBA (10% Sucrose)</td>
<td>200 mg L⁻¹</td>
<td>35.87±2.07</td>
<td>6.37±0.58</td>
</tr>
<tr>
<td>IBA (10% Sucrose)</td>
<td>300 mg L⁻¹</td>
<td>28.99±3.75</td>
<td>5.73±0.63</td>
</tr>
<tr>
<td>GA3 (10% Sucrose)</td>
<td>100 mg L⁻¹</td>
<td>34.02±1.71</td>
<td>5.63±0.66</td>
</tr>
<tr>
<td>GA3 (10% Sucrose)</td>
<td>200 mg L⁻¹</td>
<td>52.10±5.30</td>
<td>10.56±0.74</td>
</tr>
<tr>
<td>GA3 (10% Sucrose)</td>
<td>300 mg L⁻¹</td>
<td>44.48±3.26</td>
<td>6.52±1.16</td>
</tr>
<tr>
<td>Kinetin (10% Sucrose)</td>
<td>100 mg L⁻¹</td>
<td>36.06±4.04</td>
<td>6.44±0.69</td>
</tr>
<tr>
<td>Kinetin (10% Sucrose)</td>
<td>200 mg L⁻¹</td>
<td>23.37±1.67</td>
<td>4.85±0.55</td>
</tr>
<tr>
<td>Kinetin (10% Sucrose)</td>
<td>300 mg L⁻¹</td>
<td>28.96±3.08</td>
<td>5.90±0.56</td>
</tr>
</tbody>
</table>

Results are shown as Mean ± SEM

Table 2. ANOVA for the effect of hormones, concentrations, time and species

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Response variable</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clerodendrum colebrookianum</td>
<td>hormones</td>
<td>3</td>
<td>1548.28</td>
<td>7.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>concentrations</td>
<td>2</td>
<td>759.69</td>
<td>3.42</td>
<td>0.0359</td>
</tr>
<tr>
<td></td>
<td>time</td>
<td>2</td>
<td>2059.96</td>
<td>9.88</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Clerodendrum infortunatum</td>
<td>hormone</td>
<td>3</td>
<td>2492.38</td>
<td>11.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>concentrations</td>
<td>2</td>
<td>1569.22</td>
<td>6.35</td>
<td>0.0023</td>
</tr>
<tr>
<td></td>
<td>time</td>
<td>2</td>
<td>2798.12</td>
<td>11.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Between Species</td>
<td></td>
<td>1</td>
<td>0.04</td>
<td>0.07</td>
<td>0.7825*</td>
</tr>
</tbody>
</table>
tion (0.85±0.23 and 0.78±0.78, respectively for \textit{C. colebrookianum} and \textit{C. infortunatum}) in the first 24 hours and later 48 and 72 hours no germination was recorded in both species. Sucrose concentrations (5% and 10%) were found to induce \textit{in vitro} pollen germination and acted as a fundamental substrate compared to control. In 5% sucrose concentration, a low percentage of pollen germination was recorded with 5.19±0.60% in \textit{C. colebrookianum} and 10.65±1.14% in \textit{C. infortunatum} while at 10% sucrose concentration; still low germination of 7.54±1.21 was recorded for \textit{C. colebrookianum} and fair germination (17.92±4.93%) in \textit{C. infortunatum} in the initial 24 hours. Further, the germination percentage relatively declined as time passed, i.e., at 48 hours and 72 hours (Fig. 1). Significant differences (p<0.0001) were observed between distilled water and sucrose concentrations at 5% & 10% and time response (Table 4).

Maximum pollen germination was recorded in the first 24 hours, which decreased with time, i.e., 48 and 72 hours. The lowest germination percentage was observed after 72 hours in 100, 200, and 300 mg L\(^{-1}\) concentrations in all the selected growth hormones, i.e., IAA, IBA, GA\(_3\), and Kinetin in both plant species (Table 1).

In \textit{C. colebrookianum}, the highest \textit{in vitro} pollen germination of 52.10±5.30% was recorded in GA\(_3\) (200 mg

### Table 3. Pollen viability tested with TTC for two Clerodendrum species

<table>
<thead>
<tr>
<th>Species</th>
<th>\textit{C. colebrookianum}</th>
<th>\textit{C. infortunatum}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable %</td>
<td>Viable %</td>
<td></td>
</tr>
<tr>
<td>Pre anthesis</td>
<td>28.57%±2.61%</td>
<td>19.37%±1.73%</td>
</tr>
<tr>
<td>(un-open flower)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthesis</td>
<td>71.97%±4.30%</td>
<td>81.63%±3.23</td>
</tr>
<tr>
<td>(open flower)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. ANOVA of the effect of distilled water and sucrose 5% and 10% concentrations and time.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Clerodendrum colebrookianum}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control and sucrose</td>
<td>2</td>
<td>100.94</td>
<td>16.69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5% &amp; 10%</td>
<td></td>
<td>648.13</td>
<td>76.54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>511.80</td>
<td>17.57</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>\textit{Clerodendrum infortunatum}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control and sucrose</td>
<td>2</td>
<td>3640.49</td>
<td>129.81</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5% &amp; 10%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Impact of sucrose concentration on \textit{in-vitro} pollen germination in \textit{C. colebrookianum} and \textit{C. infortunatum}](image1)

![Fig. 2. Pollen storage of \textit{C. colebrookianum} under different storage temperatures (-20°C, -4°C and 6°C)](image2)
L⁻¹), followed by 46.56±4.59 % in IBA (100 mg L⁻¹), 44.48±3.26% in GA₃ (300 mg L⁻¹) and least 23.37±1.67% in Kineticin (200 mg L⁻¹). In the case of C. infortunatum, the highest in vitro pollen germination of 61.91±1.76% was also recorded in GA₃ (200 mg L⁻¹), followed by 48.61±1.79 % in IBA (200 mg L⁻¹), 43.42±2.37% in Kineticin (100 mg L⁻¹) and least 25.45±2.89% in IAA (100 mg L⁻¹). Hence GA₃ (200 mg L⁻¹) was found to be the most suitable growth hormone concentration, followed by IBA (200 mg L⁻¹ and 100 mg L⁻¹) for inducing in vitro pollen germination in both Clerodendrum species (Table 1). Statistically, the response of all the treatments viz. hormones, their concentrations and time, on in vitro pollen germination of C. colebrookianum and C. infortunatum was found to be significantly different (p<0.05) (Table 2). It was observed that among the treatments, different hormones and times gave higher significant responses (p<0.0001), followed by the hormone concentrations application (p<0.05). A non-significant difference was recorded between the study plant species for in vitro pollen germination (Table 3).

Under varied temperatures storage conditions such as -20°C, -4°C, and 6°C, pollen viability of both selected Clerodendrum species decreased with increased storage durations. It was observed that the pollen grains of both Clerodendrum species’ stored at -20°C and 6°C showed relatively longer pollen viability duration; pollen grains lost their viability after 28 days. It was also observed that the pollen which were stored under -4°C lost their viability within 14 days of storage in two selected Clerodendrum species. Hence, storage conditions of both C. colebrookianum and C. infortunatum followed a similar trend (Figures 2 and 3). There is significant difference between storage days for both studied plant species (p<0.0001).

DISCUSSION

TTC test was found to be a dependable test for assessing pollen viability in different flowering stages, such as pre-anthesis and anthesis, to distinguish between alive and dead pollen in both C. colebrookianum and C. infortunatum. A color distinction in viable pollen stained with red while non-viable pollen with no stain was observed for both Clerodendrum species (Figs. 4 C and D). The color differentiation between alive and dead pollen using TTC was observed for Jatropha curcas (Abdelgadir et al., 2012) Prunus armeniaca (Yaman and Turan, 2021), Bursera hybrids (Rico and Reyes, 2019), and Fraxinus excelsior (Buchner et al., 2022). Yang et al. (2021) found that the TTC test for pollen viability is reliable for Amomum villosum and Amomum longiligulare. The finding of a high viable percentage after anthesis in both the Clerodendrum species (Table 1), is similar to the findings observed for Jatropha curcas (Abdelgadir et al., 2012), Passiflora cincinnata, Passiflora edulis, Passiflora edmundi, Passiflora galbana, Passiflora gibertii, and Passiflora suberosa (Soares et al., 2013). Shekari et al., (2016b) observed low viable and low germination percentage of pollen for Leonurus cardiaca before anthesis. Pollen viability is often correlated with pollen quality to be used in artificial pollination and breeding (Dafni and Firmage, 2000). Selection of appropriate anthesis stage for notably viable pollen grains is paramount in pollination, fertilization, and breeding of Passiflora sp. (Soares et al., 2013); improved cultivars of banana (Soares et al., 2015) and Leonurus cardiaca (Shekari et al., 2016b).

Pollen germination under in vitro experiments help to recreate the in vivo environment of pollen tube germination on the pistil. Sucrose helps to increase pollen germination and tube growth, thereby providing nutrients to the culture media (Lin et al., 2017). Appropriate sucrose concentration is a source of nutrition, osmotic balance, and vital carbon energy to induce pollen germination (Dong and Beckles, 2019). A high sucrose concentration may inhibit pollen grain germination (Lin et al., 2017). Within the same culture and media
variations, pollen germination might occur due to unbalanced osmotic pressure (Yombi et al., 2015). Significant variation in pollen germination and tube growth was seen between the sucrose concentrations in both of the Clerodendrum species. Increased in sucrose concentration from 5 % to 10 % enhanced the pollen germination in both the Clerodendrum species (Fig. 1.). Similar result in Cunninghamia lanceolata (Fragallah et al., 2019), Psidium guajava (Sarkar et al., 2018), Impatiens balsamina (Patel and Mankad, 2015) and Leonurus cardiaca (Shekari et al., 2016 a) were recorded for the effect of sucrose concentration on percentage of pollen germination. A low concentration of sucrose gives low pollen germination, while above >10% sucrose concentration gives high pollen germination in Momordica subangulata (Naik et al., 2016). The pollen germination rates varied significantly between incubation times and between treatments. Pollen germination rates were higher at 24 hours and less at 48 hours (Table 4). The results revealed that the pollen grains of both species of Clerodendrum need just 24 hours to start growing for germination (Fig. 1). A similar effect was observed for Prunus laurocerasus (Sulusoglu and Cavusoglu, 2014); Cunninghamia lanceolata (Fragallah et al., 2019) and Spathodea campanulata, Bauhinia purpurea and B. racemosa (Sanjay et al., 2016). The rate of pollen germination determines the effectiveness of pollen germination media. All four-growth hormones influenced pollen germination, but their rate differed (Table 2 and Figs. 4. E & F). Different concentrations of hormones give different germination percentages (Table 2). An increase in the concentration of GA3 enhanced the pollen germination in the two Clerodendrum species; thus, a high concentration of GA3 plays a vital function in pollen germination. A similar result was observed for Acca sellowiana (Xiong et al., 2016), Prunus dulcis (Maita and Sotomayor, 2015), and functional male flower of pomegranate (Engin and Gokbayrak, 2016). GA3 promotes amylase activity, acid phosphatase, and β-glucosidase. It enhances the leaching of amylase and acid phosphatase enzymes to stimulate pollen germination (Sanjay et al., 2016). GA3 significantly promoted in vitro pollen germination of Vitis vinifera, Spathodea campanulata, and Momordica charantia (Gokbayrak and Engin, 2015; Sanjay et al., 2016). Kinetin regulates pollen germination and tube growth at different concentrations (Manonmani and Mekala, 2016; Marchioretto et al., 2019). A high concentration of Kinetin reduced pollen germination (Table 2) in both the Clerodendrum species, while a lower concentration of Kinetin was suitable for pollen germination and tube growth. Dziurka et al., (2019); Usman et al., (2022) reported low content of Kinetin in the plant improves regeneration, thereby increasing the efficiency of doubled haploid production; this supports our finding. Kinetin is reported to influence the germination of pollen and tube growth of Prunus dulcis (Maita and Sotomayor, 2015). In C. colebrookianum, IBA and IAA at higher concentra-
tions reduced pollen germination, but in *C. infortunatum*, IBA and IAA increased pollen germination at higher concentrations (Table 2). Thus, suitable concentrations of IAA and IBA are needed for both the *Clerodendrum* species. IBA promoted *in vitro* pollen germination in four Hibiscus species (Li et al., 2015), *B. purpurea* and *B. racemosa* (Sanjay et al., 2016), *Litchi chinensis* (Zeng et al., 2018), and *Actinidia deliciosa* pollen (Marques, 2018). Abdelgadir et al. (2012) reported that suitable IAA concentration is needed for proper pollen germination and tube growth; this supports our finding too for the two *Clerodendrum* species. In addition, Kovaleva et al. (2005) reported that low concentrations of IAA promoted pollen germination while higher concentrations inhibited it. IAA stimulated pollen germination *in vitro* of male *Petunia hybrida* through osmoregulation by activating K⁺ channels (Kovaleva et al., 2016).

IAA, GA3, IBA, and Kinetin influenced and regulated *in vitro* pollen germination for both *Clerodendrum* species. But their response in two select-ed *Clerodendrum* species differed with growth hormones and their concentrations, suggesting that the pollen grain of the two *Clerodendrum* species react differentially with different growth hormones. Long-term pollen storage is essential for plant breeding, especially in asynchronous flowering species and germplasm exchange. The longevity of pollen differs from plant species to species and from minutes to months. Thus, there is a practical need to evaluate and standardize storage conditions of pollen grains to maintain their vitality for an extended period for making crosses between two varieties/species which flower at different times. From the result of pollen storage, pollen grains of two *Clerodendrum* species which were stored (Fig. 2 and 3) at various temperatures (-20°C, -4°C and 6°C) for varying lengths of time (0-28 days) revealed that the pollen viability decreased after storage at different temperatures with an increase in the interval of time. This would be due to the fact that the metabolic activity of pollen depends on temperature and time (Du et al., 2019). Among the stored temperatures (-20°C, -4°C and 6°C), viability percentage of pollen varies significantly in both *Clerodendrum* species. Temperature and humidity influence the viability of pollen (Sidhu, 2019; Du et al., 2019). Therefore, appropriate temperature is needed to be screened through more experimentation to extend the longevity of pollen viability of *C. colebrookianum* up to 70 days (as flowering occurred between July to December) so that species hybridization could be done with *C. infortunatum* (flowering occurred between February to April 2019) to develop new species with novel importance. Pollen grains of *Fraxinus excelsior* lose their viability in warmer conditions, and they can be stored for a longer duration at temperatures of -20°C and -80°C than at 4°C; and the viability of stored pollen grains could be used to overcome crucial problem concerning ash die-back disease through future breeding program (Buchner et al., 2022). Pollen grains of *Juniperus communis* (a tree with meager seed sets due to pollen limitation under natural conditions) can be stored suitably with significant pollen viability at ~20 °C as compared to -4°C. The stored pollens were valuable for pollen supplementation experiments to enhance seed sets in the tree species (Kormutak et al., 2021). Hence pollen storage is crucial in pollen handling that can act as a valuable tool for breeders to overcome problems associated with differences in flowering time, pollen shedding, stigma receptivity, and pollen limitation in controlled pollination experiments. Further, the studies of long and short-term pollen viability and storage could help to make crosses among individuals of sub-populations growing geographically separated and adapted to biotic and abiotic gradients in racial hybridization to improve traits of interest.

**Conclusion**

It was concluded that TTC staining test is a dependable test to evaluate the viability of pollen grains for *C. colebrookianum* and *C. infortunatum*. Sucrose (5% and 10%) induces low to fair pollen germination of the pollen grains of these species. GA3 (200 mg L⁻¹) was found to be the most suitable growth hormone concentration, followed by IBA (200 mg L⁻¹ and 100 mg L⁻¹) for inducing *in vitro* pollen germination in *C. colebrookianum* and *C. infortunatum* during the first 24 hours of incubation. Among the treatments, different hormones and times gave a higher significant response (p<0.0001), followed by the hormone concentrations application (p<0.05). There was a non-significant difference between the plant species for *in vitro* pollen germination. Pollen grains of both *Clerodendrum* species remained viable up to 28 days at -20°C and 6°C. Thus, pollen grains of both *Clerodendrum* species should be collected at anthesis stage for short-term storage of pollen grains which shall be valuable for future application in pollination, supplementation, hybridization, and breeding experiments in both *Clerodendrum* species.

**ACKNOWLEDGEMENTS**

The authors are thankful for DST-FIST facilities used in the study, Department of Forestry, [No.SR/FST/LS-L/2017/65(C)], Ministry of Science & Technology, Government of India, New Delhi, India. Mr. Puyam Devdanda Singh is thankful to DBT, New Delhi for the fellowship of project fellow in the DBT sanctioned project No. BT/IN/Indo-US/Foldscope/39/2015 dated 20-03-2018.
REFERENCES


