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Research Article

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

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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 antihypertension, 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-colics 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

and Chaudhry, 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 saponin, diterpene (Clerodin), triterpene (lupeol), steroid (β-sitosterol), flavonoids, glycerides of stearic acid, linoleic acid, and lignoceric acid (Bhattacharjee *et al.,* 2011).

Compared to the medicinal importance of *C. colebrookianum* and *C. infortunatum*, there are minimal studies on its propagation (Mao *et al.,* 1995) and reproductive biology. Pollen viability, germination, and storage are essential 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 Rangswamy, 2012). *in vitro* pollen germination is used significantly on a variety 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 nutritional and physiological requirements of pollen germination 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 conservation program.

MATERIALS AND METHODS

Two species of *Clerodendrum*, viz. *C. colebrookianum* and *C. infortunatum* (Family: Lamiaceae) were selected at Tanhril village (23° 44' 15" N, 92° 39' 44" E, 748 m asl) in the district Aizawl, Mizoram. The study was conducted 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 solution 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 studies. 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 (GA_3), 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 media 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-

scopic 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, GA_3, and Kinetin), sucrose concentrations, and time in both the *Clerodendrum* species. In control (distilled water), exceptionally very low percentage of pollen germina-

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

Hormone	Concentrations	Pollen germination %					
		C. colebrookianum			*C. infortunatum*		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
IAA (10% Sucrose)	100 mg L^1	39.88±3.70	7.12±0.81	1.16±0.25	25.45±2.89	6.00±1.01	0.75±0.16
	200 mg L^1	31.80±1.97	4.73±0.61	0.42±0.15	37.02±3.21	8.25±1.39	1.46±0.24
	300 mg L^1	26.86±3.08	4.64±0.70	0.67±0.18	26.16±3.13	3.77±0.55	0.38±0.08
IBA (10% Sucrose)	100 mg L^1	46.56±4.59	9.20±0.90	2.15±0.28	34.32±3.30	6.83±0.91	1.34±0.22
	200 mg L^1	35.87±2.07	6.37±0.58	1.01±0.20	48.61±1.79	10.47±0.91	2.22±0.30
	300 mg L^1	28.99±3.75	5.73±0.63	1.40±0.23	38.27±4.83	9.21±1.40	1.80±0.24
GA_3 (10% Sucrose)	100 mg L^1	34.02±1.71	5.63±0.66	1.25±0.19	39.56±3.70	7.00±0.88	1.63±0.24
	200 mg L^1	52.10±5.30	10.56±0.74	2.03±0.20	61.91±1.76	10.44±1.12	1.92±0.33
	300 mg L^1	44.48±3.26	6.52±1.16	0.88±0.15	42.88±5.25	7.19±1.01	1.69±0.29
Kinetin (10% Sucrose)	100 mg L^1	36.06±4.04	6.44±0.69	1.50±0.21	43.42±2.37	8.87±1.45	2.02±0.35
	200 mg L^1	23.37±1.67	4.85±0.55	1.22±0.18	31.68±2.56	5.94±0.73	0.44±0.12
	300 mg L^1	28.96±3.08	5.90±0.56	1.08±0.29	37.31±4.08	6.63±0.73	1.47±0.22

Results are shown as Mean ± SEM

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

Plant species	Response variable	df	MS	F	p
Clerodendrum colebrookianum	hormones	3	1548.28	7.86	<0.0001
	concentrations	2	759.69	3.42	0.0359
	time	2	2059.96	9.88	<0.0001
Clerodendrum infortunatum	hormone	3	2492.38	11.77	<0.0001
	concentrations	2	1569.22	6.35	0.0023
	time	2	2798.12	11.67	<0.0001
Between Species		1	0.04	0.07	0.7825*

*Non-significant

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

Species	*C. colebrookianum*	*C. infortunatum*
	Viable %	Viable %
Pre anthesis (un-open flower)	28.57%±2.61%	19.37%±1.73%
Anthesis (open flower)	71.97%±4.30 %,	81.63%±3.23

tion (0.85±0.23 and 0.78±0.78, respectively for *C. colebrookianum* and *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 *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 *C. colebrookianum* and 10.65±1.14% in *C. infortunatum* while at 10% sucrose concentration; still low germination of 7.54±1.21 was recorded for *C. colebrookianum* and fair germination (17.92±4.93%) in *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 *C. colebrookianum*, the highest *in vitro* pollen germination of 52.10±5.30% was recorded in GA_3 (200 mg

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

Response variable		df	MS	F	p
Clerodendrum colebrookianum	Control and sucrose 5% & 10%	2	100.94	16.69	<0.0001
	Time	2	648.13	76.54	<0.0001
Clerodendrum infortunatum	Control and sucrose 5% & 10%	2	511.80	17.57	<0.0001
	Time	2	3640.49	129.81	<0.0001

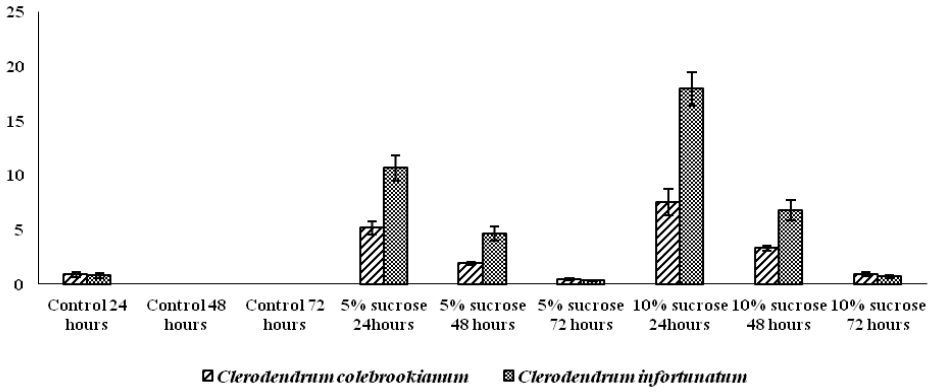

Fig. 1. *Impact of sucrose concentration on in-vitro pollen germination in C. colebrookianum and C. infortunatum*

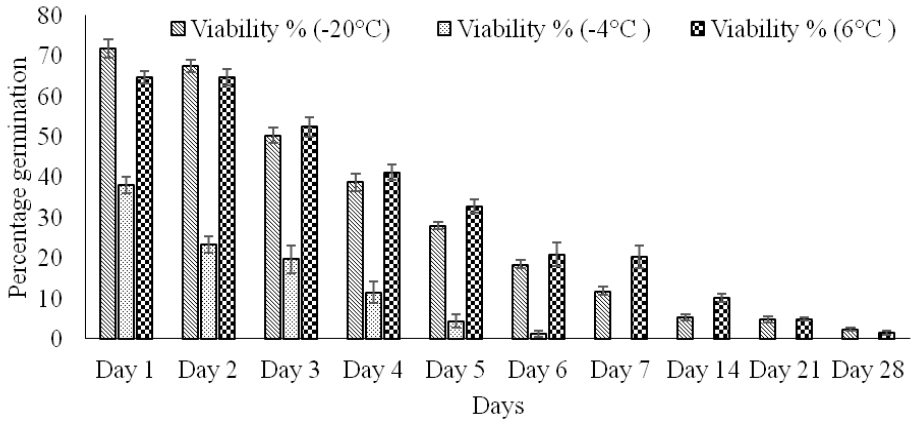

Fig. 2. *Pollen storage of C. colebrookianum under different storage temperatures (-20°C, -4°C and 6°C)*

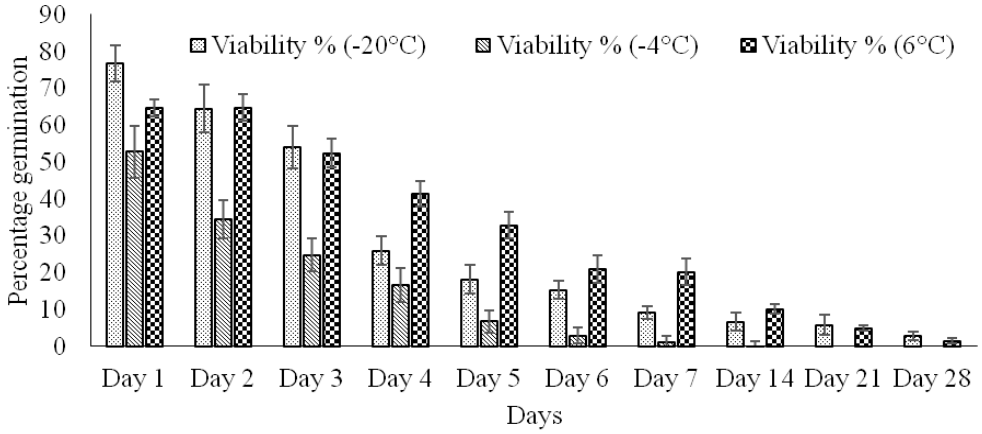

Fig. 3. *Pollen storage of Clerodendrum infortunatum under different storage temperatures (-20°C, -4°C and 6°C)*

L^{-1}), followed by 46.56±4.59 % in IBA (100 mg L^{-1}), 44.48±3.26% in GA_3 (300 mg L^{-1}) and least 23.37±1.67% in Kinetin (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 GA_3 (200 mg L^{-1}), followed by 48.61±1.79 % in IBA (200 mg L^{-1}), 43.42±2.37% in Kinetin (100 mg L^{-1}) and least 25.45±2.89% in IAA (100 mg L^{-1}). Hence GA_3 (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 pollens 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 edmundoi, 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

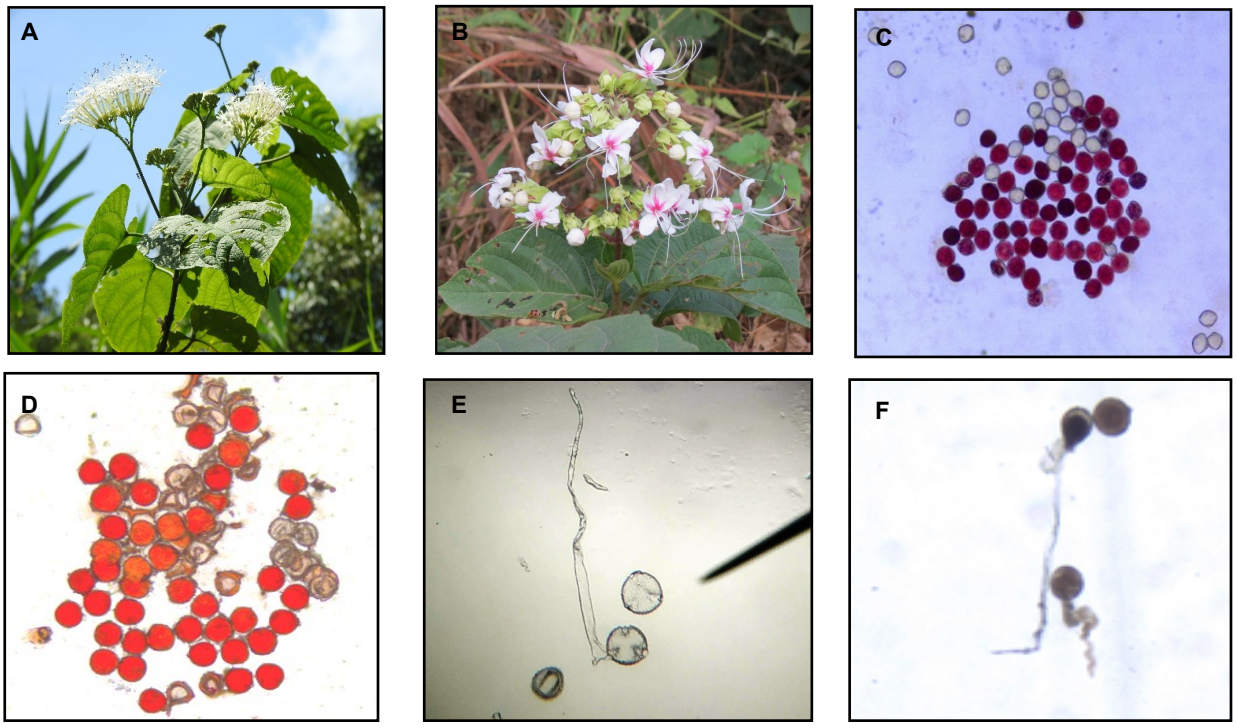

Fig. 4. (A & B): Habit of *C. colebrookianum* and *C. infortunatum*, (C& D): pollen viability in *C. colebrookianum* and *C. infortunatum*, pollen stained red is counted as viable, (E & F): *in vitro* germainating pollens of *C. colebrookianum* and *C. infortunatum*

variations, pollen germination might occur due to unbalanced osmotic pressure (Youmbi *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 *Cunnighamia 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); *Cunnighamia lanceolata* (Fragallah *et al*., 2019) and S*pathodea campanulata, Bauhinia purperea* 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 GA_3 enhanced the pollen germination in the two *Clerodendrum* species; thus, a high concentration of GA_3 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). GA_3 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). GA_3 significantly promoted *in vitro* pollen germination of *Vitis vinifera*, *Spathodea campanualata,* 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 selected *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 (0-28days) 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 dieback 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 subpopulations 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% and10%) induces low to fair pollen germination of the pollen grains of these species. GA_3 (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.

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REFERENCES

1. Abdelgadir, H. A., Johnson, S. D. & Van Staden, J. (2012). Pollen viability, pollen germination and pollen tube growth in the biofuel seed crop *Jatropha curcas* (Euphorbiaceae). *South African Journal of Botany*, *79*, 132-139. doi.org/10.1016/j.sajb.2011.10.005
2. Arya, H., Syed, S. B., Singh, S. S., Ampasala, D. R. & Coumar, M. S. (2018). In silico investigations of chemical constituents of *Clerodendrum colebrookianum* in the anti-hypertensive drug targets: Rock, Ace, and Pde5. *Interdisciplinary Sciences: Computational Life Sciences*, 10 (4), 792-804. doi.org/10.1007/s12539-017-0243-6
3. Bhattacharjee, D., Das, A., Das, S. K. & Chakraborthy, G. S. (2011). *Clerodendrum infortunatum* Linn.: A review. *J Adv Pharm Healthcare Res*, *1*(3), 82-85. http://www.japhr.iaphp.in
4. Brewbaker, J. L. & Kwack, B. H. (1963). The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany*, 50 (9), 859-865. doi.org/10.1002/j.1537-2197.1963.tb06564.x
5. Buchner, L., Eisen, A. K., Sikoparija, B. & Jochner-Oette, S. (2022). Pollen viability of fraxinus excelsior in storage experiments and investigations on the potential effect of long-range transport. *Forests*, *13* (4), 600. doi.org/10.3390/f13040600
6. Dafni, A. & Firmage, D. (2000). Pollen viability and longevity: practical, ecological and evolutionary implications. In *Pollen and pollination*, (113-132). Springer, Vienna. doi.org/10.1007/978-3-7091-6306-1_6
7. Das, A., Chaudhuri, D., Ghate, N. B., Panja, S., Chatterjee, A. & Mandal, N. (2015). Protective effect of *Clerodendrum colebrookianum* leaves against iron-induced oxidative stress and hepatotoxicity in Swiss albino mice. *Indian Journal of Experimental Biology*, 53, 281-291. nopr.niscpr.res.in/handle/123456789/31439
8. Dong, S. & Beckles, D. M. (2019). Dynamic changes in the starch-sugar interconversion within plant source and sink tissues promote a better abiotic stress response. *Journal of Plant Physiology*, 234, 80-93. doi.org/10.1016/j.jplph.2019.01.007
9. Du, G., Xu, J., Gao, C., Lu, J., Li, Q., Du, J. & Sun, X. (2019). Effect of low storage temperature on pollen viability of fifteen herbaceous peonies. *Biotechnology Reports*, *21*, e00309. doi.org/10.1016/j.btre.2019.e00309
10. Dziurka, K., Dziurka, M., Warchoł, M., Czyczyło-Mysza, I., Marcińska, I., Noga, A., Kamila, K. & Skrzypek, E. (2019). Endogenous phytohormone profile during oat (*Avena sativa* L.) haploid embryo development. *In Vitro Cellular & Developmental Biology-Plant*, *55*(2), 221-229. doi.org/10.1007/s11627-019-09967-5
11. Engin, H. & Gokbayrak, Z. (2016). Effects of brassinosteroid, naphthalene acetic acid and gibberellic acid on in vitro pollen germination of bisexual and functional male flowers of pomegranate cultivars. *COMU Ziraat Fakültesi Dergisi*, *45*.
12. Fragallah, S. A. D. A., Lin, S., Li, N., Ligate, E. J. & Chen, Y. (2019). Effects of sucrose, boric acid, pH, and incubation time on in vitro germination of pollen and tube growth of chinese fir (*Cunnighamial lanceolata* L.). *Forests*, *10* (2), 102. doi.org/10.3390/f10020102
13. Gogoi, P. & Nath, N. (2021). Indigenous knowledge of ethnomedicinal plants by the Assamese community in Dibrugarh District, Assam, India. *Journal of Threatened Taxa*, 13 (5), 18297-18312. orcid.org/0000-0003-3429-6252
14. Gokbayrak, Z. & Engin, H. A. K. A. N. (2015). Effect of plant growth regulators on in vitro pollen germination of grapevine cultivars. In *III Balkan Symposium on Fruit Growing* 1139 (405-408). doi.org/10.17660/ActaHortic.2016.1139.70
15. Hao, Q., Xu, L., Wang, H., Liu, Q. & Wang, K. (2022). Evaluation of pollen viability, stigma receptivity, and the cross barrier between tropical and hardy water lily cultivars. *Flora*, 290, 152046. doi.org/10.1016/j.flora.2022.152046
16. Helen, R. L., Jayesh, K., Syama, S. & Latha, M. S. (2021). Secondary metabolites from *Clerodendrum Infortunatum* L.: their bioactivities and health benefits. In *Health Benefits of Secondary Phytocompounds from Plant and Marine Sources* (pp. 39-60). 1st Ed. New York, Apple Academic Press. doi.org/10.1201/9781003019602
17. Jamir, K. & Tsurho, K. (2016). Documentation of medicinal plants and its uses by Phom tribe of Longleng district, Nagaland. *Journal of Medicinal Plant Studies*, *4*(6), 167-172.
18. Jaskani, M. J. & Naqvi, S. A. (2017). Storage and viability assessment of date palm pollen. In *Date Palm Biotechnology Protocols Volume II* (3-13). Humana Press, New York, NY. doi.org/10.1007/978-1-4939-7159-6_1
19. Kalita, J., Singh, S. S. & Khan, M. L. (2012). *Clerodendrum colebrookianum* Walp.: A potential folk medicinal plant of North East India. *Asian Journal of Pharmaceutical & Biological Research, 2*(4), 256-261.
20. Kormutak, A., Bolecek, P., Galgóci, M. & Gomory, D. (2021). Longevity and germination of *Juniperus communis* L. pollen after storage. *Scientific Reports*, *11*(1), 1-8. doi.org/10.1038/s41598-021-90942-9
21. Kovaleva, L. V., Voronkov, A. S., Zakharova, E. V., Minkina, Y. V., Timofeeva, G. V. & Andreev, I. M. (2016). Exogenous IAA and ABA stimulate germination of petunia male gametophyte by activating Ca2+-dependent K+-channels and by modulating the activity of plasmalemma H+-ATPase and actin cytoskeleton. *Russian Journal of Developmental Biology*, *47*(3), 109-121. doi.org/10.1134/S1062360416030036
22. Kovaleva, L. V., Zakharova, E. V., Minkina, Y. V., Timofeeva, G. V. & Andreev, I. M. (2005). Germination and in vitro growth of petunia male gametophyte are affected by exogenous hormones and involve the changes in the endogenous hormone level. *Russian Journal of Plant Physiology*, *52*(4), 521-526. doi.org/10.1007/s11183-005-0077-7
23. Kshetri, P., Tamreihao, K., Roy, S. S., Singh, T. S., Sharma, S. K. & Ansari, M. A. (2022). Ethnomedicinal plants of north eastern Himalayan region of India to combat hypertension. In *Medicinal Plants* (pp. 461-483). 1st Ed. New York, Apple Academic Press. doi.org/10.1201/9781003277408
24. Li, Y., Tang, G. & Luo, F. (2015). Studies on pollen viability and cross-compatibility of four species of Hibiscus. *Journal of Nanjing Forestry University (Natural Sciences Edition)*, *39*(2), 183-186. doi.org/10.3969/

j.issn.1000-2006.2015.02.032
25. Lin, Y., Wang, Y., Iqbal, A., Shi, P., Li, J., Yang, Y. & Lei, X. (2017). Optimization of culture medium and temperature for the in vitro germination of oil palm pollen. *Scientia Horticulturae*, 220, 134-138. doi.org/10.1016/j.scienta.2017.03.040
26. Lokesh, D. & Amitsankar, D. (2012). Evaluation of mechanism for antihypertensive action of *Clerodendrum colebrookianum* Walp., used by folklore healers in north-east India. *Journal of Ethnopharmacology*, *143*(1), 207-212. doi.org/10.1016/j.jep.2012.06.025
27. Maita, S. & Sotomayor, C. (2015). The effect of three plant bioregulators on pollen germination, pollen tube growth and fruit set in almond [*Prunus dulcis* (Mill.) DA Webb] cvs. Non Pareil and Carmel. *Electronic Journal of Biotechnology*, *18*(5), 381-386. doi.org/10.1016/j.ejbt.2015.07.004
28. Manonmani, R. & Mekala, R. (2016). Pollen morphology, viability, germination and tube growth of some plant species. *Ecology*, *3*(4), 5-6. doi.10.20959/wjpr20167-6437
29. Mao, A. A., Wetten, A., Fay, M. & Caligari, P. D. S. (1995). In vitro propagation of *Clerodendrum colebrookianum* Walp., a potential natural anti-hypertension medicinal plant. *Plant Cell Reports*, *14*(8), 493-496. doi.org/10.1007/BF00232781
30. Marchioretto, L. D. R., De Rossi, A., do Amaral, L. O. & de Souza Ribeiro, A. M. A. (2019). Efficacy and mode of action of blossom thinners on 'Fuji More'apple trees. *Scientia Horticulturae*, *246*, 634-642. doi.org/10.1016/j.scienta.2018.11.039
31. Marques, J. E. N. (2018). *Assays of In vitro germination of kiwi pollen* (Doctoral dissertation, Universidade de Coimbra). hdl.handle.net/10316/86144
32. Mesnoua, M., Roumani, M. & Salem, A. (2018). The effect of pollen storage temperatures on pollen viability, fruit set and fruit quality of six date palm cultivars. *Scientia Horticulturae*, *236*, 279-283. doi.org/10.1016/j.scienta.2018.03.053
33. Murtem, G. & Chaudhry, P. (2016). An ethnobotanical study of medicinal plants used by the tribes in upper Subansiri district of Arunachal Pradesh, India. *American journal of ethnomedicine*, *3*(3), 35-49. www.ajethno.com
34. Naik, A., Akhtar, S., Chattopadhyay, A., Thapa, U. & Hazra, P. (2016). In vitro teasle gourd pollen germination and pollen tube development as affected by sucrose, boric acid, and inorganic salts. *International Journal of Vegetable Science*, *22*(2), 209-216. doi.org/10.1080/19315260.2015.1008665
35. Nandi, S. & Mawkhlieng Lyndem, L. (2016). *Clerodendrum viscosum*: traditional uses, pharmacological activities and phytochemical constituents. *Natural Product Research*, 30(5), 497-506. doi.org/10.1080/14786419.2015.1025229
36. Patel, E. & Mankad, A. (2015). Sucrose needs for pollen germination of *Impatiens balsamina* L. *International Journal of Innovative Research in Sci, Eng. and Tech*, *4*(10), 10242-10244. doi:10.15680/IJIRSET.2015.0410104
37. Rej, S., Dutta, M., Jamal, S., Das, S. & Chatterjee, S. (2014). Study of phytochemical constituents and antibacterial activity of *Clerodendrum infortunatum*. *Asian Journal of Research in Pharmaceutical Science*, *4*(4), 187-195.
38. Rico, Y. & Reyes-Estanislao, L. (2019). Pollen viability and germinability of putative Bursera hybrids (section Bullockia; Burseraceae) in Mexico. *Acta Botánica Mexicana*, (126). doi.org/10.21829/abm126.2019.1435
39. Saha, S., Mukherjee, A., Biswas, S., Choudhury, D., Saha, J., Pal, S., Sarkar, M. & Kole, R. K. (2018). Formulation and chemical characterization of *Clerodendrum infortunatum* leaf extract in relation to anti-fungal activity. *Heliyon*, *4*(12), e01047. doi.org/10.1016/j.heliyon.2018.e01047
40. Sanjay Kumar, K., Prasad Khanduri, V., Kar, K., Mohan Sharma, C. & Kumar Riyal, M. (2016). Effect of growth regulators and time on in vitro pollen germination in three ornamental tropical tree species. *Journal of Agricultural Science and Technology*, *18*(5), 1247-1255. doi.jast.modares.ac.ir/article-23-2964-en.html
41. Sarkar, T., Sarkar, S. K. & Vangaru, S. (2018). Effect of sucrose and boric acid on in-vitro pollen germination of guava (*Psidium guajava*) varieties. *Advances Research*, *15*, 1-9. doi:10.9734/AIR/2018/41145
42. Shekari, A., Nazeri, V. & Shokrpour, M. (2016 a). Influence of boric acid, sucrose and temperature on the germination of *Leonurus cardiaca* L., pollen. *International Journal of Farming and Allied Sciences, 5*, 363-366.
43. Shekari, A., Nazeri, V. & Shokrpour, M. (2016 b). Pollen viability and storage life in *Leonurus cardiaca* L. *Journal of Applied Research on Medicinal and Aromatic Plants*, *3*(3), 101-104. doi.org/10.1016/j.jarmap.2016.02.004
44. Shivanna, K. R. (2019). *Pollen biology and biotechnology*, 316. CRC Press, 1 edition, Boca Raton. doi.org/10.1201/9780429187704
45. Shivanna, K. R. & Rangaswamy, N. S. (2012). *Pollen biology: a laboratory manual*. Springer Science & Business Media.
46. Sidhu, R. K. (2019). Pollen storage in vegetable crops: a review. *Journal of Pharmacognosy and Phytochemistry, SP1*, 599-603.
47. Soares, T. L., de Souza, E. H., Sampaio, L. F. S., de Carvalho Costa, M. A. P., e Silva, S. D. O. & dos Santos-Sejero, J. A. (2015). Effect of collection time on the viability of banana pollen grains. *African Journal of Biotechnology*, *14*(14), 1207-1214. doi.org/10.5897/AJB2015.14449
48. Soares, T. L., Jesus, O. N. D., Santos-Serejo, J. A. D. & Oliveira, E. J. D. (2013). In vitro pollen germination and pollen viability in passion fruit (*Passiflora* spp.). *Revista Brasileira de Fruticultura*, 35 (4), 1116-1126. doi.org/10.1590/S0100-29452013000400023
49. Sulusoglu, M. & Cavusoglu, A. (2014). In vitro pollen viability and pollen germination in cherry laurel (*Prunus laurocerasus* L.). *The Scientific World Journal*, *2014*. doi.org/10.1155/2014/657123
50. Tuinstra, M. R. & Wedel, J. (2000). Estimation of pollen viability in grain sorghum. *Crop Science*, 40 (4), 968-970. doi.org/10.2135/cropsci2000.404968x
51. Usman, M., Khan, M. S., Khan, M. M., Jaskani, M. J. & Khan, A. S. (2022). Development of an efficient and reproducible in vitro regeneration system in carrot (T-29). *Pakistan Journal of Botany*, 54(3), 943-951. doi.org/10.30848/PJB2022-3(10)
52. Xiong, H., Zou, F., Yuan, D., Zhang, X. & Tan, X. (2016). Orthogonal test design for optimising the culture medium for in vitro pollen germination of feijoa (*Acca sellowiana* cv. Unique). *New Zealand Journal of Crop and Horticultural Science*, *44*(3), 192-202. doi.org/10.1080/01140671.2

016.1174945

53. Yadav, A., Verma, P. K., Chand, T. & Bora, H. R. (2018). Ethno-medicinal knowledge of *Clerodendrum* L. among different tribes of Nambor reserve forest, Assam, India. *Journal of Pharmacognosy and Phytochemistry, 7*(5), 1567-1570.
54. Yaman, M. & Turan, S. (2021). Determination of pollen viability and germination ratios in mersin ecology of some early apricot. *Current Trends in Natural Sciences*, *10*(19), 166-169. doi.org/10.47068/ctns.2021.v10i19.021
55. Yang, H., Jiang, B., Hou, A. J., Lin, Z. W. & Sun, H. D. (2000). Colebroside A, a new diglucoside of fatty acid ester of glycerin from *Clerodendrum colebrookianum*. *Journal of Asian Natural Products Research*, 2 (3), 177-185. doi.org/10.1080/10286020008039909
56. Yang, R., Wang, J., Gao, W., Jiang, Y., Su, J., Sun, D. & He, G. (2021). Research on the reproductive biological characteristics of *Amomum villosum* Lour. and *Amomum longiligulare* TL Wu. *Plos One*, *16*(8), e0250335. doi.org/10.1371/journal.pone.0250335
57. Youmbi, E., Tabi, K., Ebongue, N., Frank, G., Tonfack, L. B. & Ntsomboh, G. (2015). Oil palm (*Elaeis guineensis* Jacq.) improvement: Pollen assessment for better conservation and germination. *Journal of Oil Palm Research, 27* (3), 212-9.
58. Zeng, L., Gao, M. & Liao, W. (2018). Effects of Asc and IBA on germination and growth of Nuomici litchi pollen in different batches. *Journal of Southern Agriculture*, *49*(2), 301-306.