Studies on pollen viability and *in vitro* pollen germination of *Clerodendrum inerme* (L.) Gaertn

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Abstract

Pollen viability and *in vitro* pollen germination of *Clerodendrum inerme* (L.) Gaertn. of the family Verbenaceae has been carried out to determine the viability and fertility of pollen grains for effective breeding programme. Pollen grains of the flower of second day after anthesis showed maximum 91% pollen viability in 1% acetocarmine test at around 17.00 h. Role of sucrose (C\(_{12}H_{22}O_{11}\)) and boric acid (H\(_3\)BO\(_3\)) in different concentrations singly or in combination on pollen germination as well as pollen tube elongation has been analyzed to determine the biochemistry of pollen grains for successful fertilization. Different concentrations of sucrose respond in pollen germination. Maximum 89% germinating pollen with a mean 1800±998.88 \(\mu\)m long pollen tube development was recorded in 12% sucrose. But addition of boric acid facilitates an increased pollen germination as well as pollen tube elongation. Best 90% pollen germination along with 3270±844.65 \(\mu\)m pollen tube elongation was observed when sucrose solution (12%) was supplemented with boric acid (100 \(\mu\)g/ml). The pollen grains collected from the dehisced anther of the flower of first day anthesis at around 17.00 h showed the best germination but gradually decreased in course of time and ultimately becomes nil during drooping stage. The results revealed that boric acid play an important role on *in vitro* pollen germination and pollen tube elongation in the investigated plant taxa. Thus *in vitro* pollen germination is used in comparison with the stain technique as a confirmatory test to determine the viability as well as fertility of the pollen grains. Pollen grains retain their longest viability in three consecutive days after anthesis for greater success of pollination in *C inerme*.

Key Words: *Clerodendrum inerme* (L.) Gaertn., *in vitro* pollen germination, pollen fertility, pollen tube elongation, pollen viability, pollination.

Introduction

*Clerodendrum inerme* (L.) Gaertn. (Figure 1A) is commonly known as Smooth Volkameria (Khare, 2007) and is characterized by the appearance of white flower (Figure 1B). The plant has high medicinal value and exhibits analgesic as well as antimicrobial properties (Khare, 2007). Leaves are used in relieving muscular pains and stiffness of legs (Khare, 2007). The plants are bisexual and their fruit set principally depends on dissemination of pollen grains (Raju and Kumar, 2016). But viability and fertility of the pollen grains are the key factors for successful fertilization. Germinating pollen grain acts as male gametophyte and a prerequisite for fertilization to yield seed (Biswa and Mondal, 2014\(^a\)). Thus a successful post pollination event is not only based on stigma receptivity but also on viability as well as fertility of pollen grains. Receptive surface of the...
stigma is the proper place for *in vivo* pollen germination and pollen tube elongation, because biochemical exudation of stigmatic tissues stimulates pollen germination as well as guides pollen tube to reach in the ovule for fertilization (Biswas and Mohammad, 2016). The information of biochemistry and physiology of pollen germination and pollen tube growth are not feasible on *in vivo* studies due to involvement in complexity of the pistillate and other tissues in the stigmatic surface (Biswas, 2017). Determination of viability of pollen using different stains is quick and easy, which is used to distinguish between fertile and sterile pollen. Information about pollen fertility i.e. germination ability of pollen grains principally comes from the *in vitro* studies, because all viable pollen may not have fertility. Biochemical information on pollen viability and fertility is not only important for fruit set but also for flower-pollinator interactions and determination of breeding strategies. The pollen tube after reaching the embryo sac releases sperms for the events of fertilization. Pollen tube grows through tip extension and can be stimulated by several factors and nutrients. Thus it is necessary to know the pollen viability, fertility and pollen tube elongation before going to an effective breeding programme (Biswas and Mondal, 2014). The present work is carried out to determine the viability and fertility of pollen grains as well as to investigate the effect of sucrose and boric acid separately or in combination on *in vitro* pollen germination and pollen tube elongation of *Clerodendrum inerme* (L.) Gaertn. belonging to the family Verbenaceae.

**Materials and Methods**

Pollen viability of *C. inerme* was determined using 1% acetocarmine dissolved in glycerine as well as 0.1% TTC (2, 3, 5-triphenyl tetrazolium chloride) mounted in 50% sucrose solution following the method of Shivanna and Rangaswamy (1993). The scoring of viability was measured according to the intensity of colour of pollen grains after 30 min of incubation at dark humid chamber under controlled laboratory temperature (30±2°C). Reddish-brown colour pollen was considered as viable and yellow or colourless pollen as non-viable.

*In vitro* pollen germination of *C. inerme* was conducted to determine the effect of different nutrients like sucrose (C_{12}H_{22}O_{11}) and boric acid (H_{2}BO_{3}) at various concentrations. Different concentrations of sucrose (1-40%) and boric acid (25-500 µg/ml) were prepared and used individually or in combination. 50 µl of each solution was poured into the groove of grooved slides either individually or in combination. The pollen grains were collected from the dehisced anther of all three forms of flower after anthesis (Figure 1B) and suspended into the above mentioned nutrients with the help of aseptic platinum needle. All the slides were then placed in petri dishes, lined with moist filter paper for incubation. After stipulated period of incubation all the slides were observed under light microscope (Olympus, Model no. CH20i B1MF) at low (10x×10x) as well as high (10x×40x) magnification. All experiments were performed in triplicate. Results were tabulated and analyzed following the procedure of Shivanna and Rangaswamy (1993).

According to Dafni (2000) pollen grain was considered to be germinated when tube length exceeded beyond the diameter of pollen grain.
Results

In *C. inerme* flower anthesis occurred at evening (16.30-17.30 h). Starting from anthesis, three forms of flowers are noticed which stayed for three consecutive days (Figure 1B). Pollen grains of the investigated taxa responded in viability test (Table 1). The viable pollen showed reddish-brown colour and non-viable pollen remained colourless in 0.1% tetrazolium test (TTC) and 1% acetocarmine test (Figure 2A-B). Increased pollen viability was observed in 1% acetocarmine test than that of the 0.1% tetrazolium test (Table 1). Pollen grains of the flower of 2nd day after anthesis showed maximum 91% pollen viability in 1% acetocarmine test at evening around 17.00 h (Table 1; Figure 2A).

*In vitro* pollen germination showed 89% germinating pollen with a mean of 1800±998.88 μm long pollen tube in 12% sucrose (Table 2; Figure 2C-D). Individually boric acid showed no pollen germination. The best pollen germination (90%) with a mean of 3270±844.65 μm long pollen tube occurred after 8 h incubation in 12% sucrose supplemented with 100 μg/ml boric acid solution (Table 3; Figure 2E-F). The pollen grains collected from dehisced anthers of the flower of 1st day of anthesis at around 17.00 h showed the best germination but gradually decreased in course of time and ultimately becomes nil during drooping stage.
Table 1: Pollen viability of *Clerodendrum inerme*

<table>
<thead>
<tr>
<th>Day and Time</th>
<th>Pollen viability (%)</th>
<th>1% Acetocarmine</th>
<th>0.1% TTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^{st}) day of anthesis (Evening, 16.30-17.30 h)</td>
<td>86</td>
<td></td>
<td>69</td>
</tr>
<tr>
<td>2(^{nd}) day of anthesis (Morning, 8.00-10.00 h)</td>
<td>89</td>
<td></td>
<td>74</td>
</tr>
<tr>
<td>2(^{rd}) day of anthesis (Evening, 16.30-17.30 h)</td>
<td>91</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>3(^{rd}) day of anthesis (Drooping stage)</td>
<td>82</td>
<td></td>
<td>65</td>
</tr>
</tbody>
</table>

Table 2: Effect of sucrose on \textit{in vitro} pollen germination of *Clerodendrum inerme*

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>After 1 h</th>
<th>After 4 h</th>
<th>After 8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germination (%)</td>
<td>Tube length (µm)</td>
<td>Germination (%)</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>124±49.71 (R 50-200, N=10)</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>32</td>
<td>248±195.03 (R 50-600, N=10)</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>605±357.80 (R 150-1200, N=10)</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>68</td>
<td>785±251.71 (R 350-1200, N=10)</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>82</td>
<td>1390±391.51 (R 900-2500, N=10)</td>
<td>86</td>
</tr>
<tr>
<td>Concentration (%) + (µg/ml)</td>
<td>After 1 h</td>
<td>After 4 h</td>
<td>After 8 h</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>Germination (%)</td>
<td>Tube length (µm)</td>
<td>Germination (%)</td>
</tr>
<tr>
<td>12+25</td>
<td>34</td>
<td>1620±500.66 (R 1000-2400, N=10)</td>
<td>42</td>
</tr>
<tr>
<td>12+50</td>
<td>46</td>
<td>1620±439.19 (R 1100-2500, N=10)</td>
<td>52</td>
</tr>
<tr>
<td>12+100</td>
<td>82</td>
<td>2400±274.87 (R 2100-2800, N=10)</td>
<td>87</td>
</tr>
<tr>
<td>12+200</td>
<td>53</td>
<td>1650±516.93 (R 1000-2300, N=10)</td>
<td>66</td>
</tr>
</tbody>
</table>

± Standard deviation, N= Number of observation, R= Range

Table 3: Effect of sucrose and boric acid on in vitro pollen germination of Clerodendrum inerme
<table>
<thead>
<tr>
<th>Value</th>
<th>N</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>N</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>12+300</td>
<td>45</td>
<td>1370±211.08</td>
<td>(R 1000-1600, N=10)</td>
<td>50</td>
<td>1450±337.47</td>
<td>(R 1000-2200, N=10)</td>
</tr>
<tr>
<td>12+400</td>
<td>34</td>
<td>1190±288.48</td>
<td>(R 700-1600, N=10)</td>
<td>42</td>
<td>1440±340.58</td>
<td>(R 1000-2000, N=10)</td>
</tr>
<tr>
<td>12+500</td>
<td>22</td>
<td>1220±239.44</td>
<td>(R 700-1500, N=10)</td>
<td>30</td>
<td>1400±362.09</td>
<td>(R 1000-2000, N=10)</td>
</tr>
</tbody>
</table>

± Standard deviation, N= Number of observation, R= Range
Figure 1. Clerodendrum inerme: A. Plant habit and B. Three forms of flower (1st, 2nd and 3rd day old) after anthesis.
Figure 2. *Clerodendrum inerme*: A. Acetocarmine test showing viable pollen grains (VPG) and non-viable pollen grains (NVPG) (LM 40×), B. Tetrazolium test showing viable pollen grains (VPG) (LM 40×) C-D. *In vitro* pollen germination showing pollen tube (PT) in sucrose solution and E-F. *In vitro* pollen germination showing pollen tube (PT) in sucrose solution supplemented with boric acid.

VPG= Viable pollen grain, NVPG=Non-viable pollen grain, PT=Pollen tube, LM=Light Microscope.
Discussion

The *in vitro* effect of sucrose suggests that sucrose has an increasing influence in pollen germination which is directly proportional to the concentrations of sucrose up to 12% beyond that the germination percentage decreases in *C. inerme* (Table 2; Figure 2C-D). But, if boric acid is supplemented with sucrose, the germination of pollen and pollen tube elongation are increased. The best 90% germination along with 3270±844.65 μm pollen tube growths occurs in 12% sucrose supplemented with 100 μg/ml H₃BO₃ solution, (Table 3, Figure 2E-F). This is attributed to the fact that sucrose acts as substrate for proper pollen nutrition and also functions as osmoregulator, but boron may enhance the sucrose uptake and stimulate germinating ability. This is due to formation of a borate-sucrose complex, which acts as better traslocator than non borate- sucrose complex (Vasil, 1960). Combined effect of sucrose and boric acid on promising trends of pollen germination might reflect the views of Johri and Vasil (1960). Metabolic role of boron on *in vitro* pollen germination as well as pollen tube development was reported by Sidhu and Malik (1986) and Wang *et al.* (2003). It was reported that pollen grains of many economically important plant species germinate in boric acid (Subramanyam, 1989 and Vasil, 1960). Moreover, it was observed that boron deficiency causes morphological abnormalities of pollen tube which includes swelling of the tip as well as thickness of pollen tube when grown in sucrose solution individually (Figure 2D). Similar findings have been reported in several angiospermic species (Dickinson, 1978; Yang *et al.*, 1999). Dickinson (1978), Jackson (1989), Potts and Marsden-Smedley (1989), Polster *et al.* (1992) and Biswas and Mondal (2014) reported the morphological effects of boron in angiosperms during development of pollen tube. Biochemical investigation revealed that such swelling and thickness of pollen tube is due to accumulation of polysaccharide components, callose and pectin. Boron may directly or indirectly influence the synthesis of callose and affecting its distribution (Goldbach and Amberger, 1986). But Stepka *et al.* (2000) stated that pectin controls development of pollen tube. Moreover, boron is involved in pectin synthesis and thereby necessary for the development of membrane of pollen tube (Stanley and Loewus, 1964). Acidic pectin may enhance tube strength and decrease extensibility of the pollen tube wall by accumulation of Ca²⁺ (Li *et al.*, 1994; Koyama *et al.*, 2001). Sawidis and Reiss (1995) and Taylor and Hepler (1997) reported that pollen germination and pollen tube growth may be affected by many factors which include temperature, availability of calcium, zinc, boron etc.

Similar findings of *in vitro* pollen germination and pollen tube elongation were documented in angiosperms by Steer and Steer (1989), Mondal *et al.* (1991), Demeke and Hughes (1991), Kaliamoorthy *et al.* (1996), Bhattacharya and Mandal (1999), Biswas *et al.* (2008), Mondal and Ghanta (2012), Biswas and Mondal (2014a) and Biswas and Mondal (2014b). But this is the first report that sucrose at 12% supplemented with 100 μg/ml H₃BO₃ is the best medium for pollen viability as well as fertility test in *C. inerme*.

Stain technique revealed that maximum pollen viability is occurred during 2nd day of anthesis (Table 1). But *in vitro* pollen germination showed maximum pollen viability as well as fertility during 1st day of anthesis because all the viable pollen may not have the fertility (Biswas and Mondal, 2018). Flowers stayed for three consecutive days after anthesis and pollen grains retain their longest viability in three consecutive days after anthesis for greater success of pollination in *C inerme*. Thus present study showed that pollen viability tests are desired but prior testing of...
several stains on the species in question should be done using an in vitro pollen germination test as a comparison (Firmage and Dafni, 2001).

**Conclusion**

Pollen viability and fertility are important criteria for effective plant breeding programme. The viable pollen is a prerequisite for successful post pollination event, which ultimately results in high crop yields. Biochemistry as well as physiology of pollen viability and fertility largely comes from responses of pollen in stain technique and in vitro pollen germination. In vitro pollen germination test is used as a comparison beside stain technique to determine the viability as well as fertility of the pollen grain. Retaining of longest viability of pollen grains in three consecutive days after anthesis for greater success of pollination in *C inerme*. In *C. inerme* increased pollen germination as well as pollen tube elongation was due to sucrose solution \((C_{12}H_{22}O_{11})\) supplemented with boric acid \((H_3BO_3)\). Increased pollen germination and pollen tube elongation is due to formation of sugar-borate complex which acts as a better translocator for proper nutrition and osmoregulation than that of non-borate complex. Thus in *C. inerme*, swelling of pollen tube in sucrose solution is perpetuated by the application of boric acid, which forms sugar borate complex and ultimately synthesizes the pectin and its subsequent distribution for pollen tube elongation.

**References**


