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Evaluation of the cytotoxicity of the insecticide profen super by pollen germination assay

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Abstract
The yield of cultivated plants is greatly reduced due to attack by different kinds of pests. Application of pesticides to destroy the pests is a common agricultural practice. The *in vitro* pollen germination assay has been used to demonstrate the toxicity of many chemical compounds. As pollen cultures do not require aseptic conditions and are easy to set up, pollen grains offer a sensitive and easy assay system for evaluating the cytotoxicity of chemicals which can be incorporated in the germination medium. The parameters used to indicate cytotoxicity of the chemical are inhibition of germination of pollen and elongation of pollen tube. In the present study, the cytotoxicity of Profen Super, an insecticide, was assessed by performing the pollen germination assay in two plant species namely, *Catharanthus roseus* (L.) G. Don. and *Clitoria ternatea* L. Four dilutions (1:1, 1:2, 1:4 and 1:8) of the recommended dose of Profen Super were added in the germination medium used to culture pollen grains. The results clearly show that all tested dilutions of this insecticide are cytotoxic. Thus, the use of this insecticide should be avoided or a safe concentration should be determined which would allow fruit and seed set simultaneously with eradication of the pest.

Keywords: Profen Super, cytotoxicity, pollen grains, *in vitro* pollen germination, pollen tube length

Introduction

Cultivated crops are susceptible to attack by various kinds of pests including insects which can drastically reduce primary productivity and the common approach to prevent it is chemical control. Although known to have several negative effects, treatment with pesticides continues to be an essential strategy for pest eradication in agriculture. The term pesticide includes insecticide, fungicide, herbicide, nematicide, molluscicide and rodenticide (Bernardes *et al*., 2015) [1]. Pesticides are applied on the crops in the field as well as during post-harvest storage of grains (Sharma *et al*. 2019) [2]. Pesticides may persist in soil and water, and be carried to non-target area via leaching, adsorption and surface runoff (Tudi *et al*. 2021; Pathak *et al*. 2022) [3, 4]. Insecticides are a class of commonly used pesticides. The insecticides are chiefly grouped into organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids (Mota *et al*. 2022) [5].

There is sufficient evidence to indicate that pesticides are harmful to human beings and other organisms (Aktar *et al*. 2009) [6]. Many pesticides have been implicated in neurodegenerative diseases (Singh & Gautam 2021) [7]. Pesticide residue which accumulates on fruits and vegetables can be extremely toxic and poses a health hazard to humans; cancer and birth defects may occur as a consequence of prenatal exposure to pesticides (Ferreti *et al* 2007) [8]. In plants, pesticide toxicity leads to decrease in chlorophyll and protein content causing reduction of photosynthetic rate and decrease in crop yield (Sharma *et al*. 2019; Giménez–Moolhuyzen *et al*. 2020) [2,9]. Pesticide use may directly eliminate insect pollinators and the absence of sufficient pollinators in turn may indirectly affect crops (Dwivedi *et al*. 2022) [10].

Pollen grains have been utilised as a reliable and sensitive test system to biomonitor air pollution resulting from heavy metals and to investigate the effects of insecticides, pesticides, fungicides, herbicides, pollutants and toxic substances (Gentile *et al*., 1971; 1973; 1978; Bilderback, 1981; Sutherland *et al*., 1984; Nikolov *et al*., 2000; Kamble, 2005; Mehri *et al*., 2006; Salgare, 2006; Wang *et al*. 2015, Meshram and Chaturvedi, 2017) [11-21]. Screening different pesticides for their effect on germination of pollen grains, and hence on fruit- and seed-set, is essential before their application in the fields because chemical pesticides can be very harmful (Padilla *et al*., 2017) [22]. For assessment of the effects of toxic compounds and monitoring pollution, *in vitro* pollen germination and pollen tube growth have proved to be easy and sensitive methods (Shivanna and Rangaswamy, 1992; Shivanna, 2003) [23, 24]. Aseptic conditions are not required for these methods and their results are rapid. Inhibition of germination of pollen and elongation of pollen tube by a toxic compound incorporated into *in vitro* pollen cultures is indicative of its cytotoxicity (Pavlík and Jandurová, 2000; Subramanyan *et al*., 2023) [25, 26].

In the current investigation, the pollen germination assay was performed to study the effect of Profen Super EC (Emulsifiable Concentrate), a synthetic broad spectrum insecticide that is easily available in local seed shops. Profen Super (40% Profenofos + 4% Cypermethrin), an

organophosphate, is commonly employed in the control of bollworm complex pest of cotton though it can also be sprayed on garden plants, fruits and vegetables. The mechanism of action of Profen Super, also called Profex Super, involves inhibition of the enzyme acetylcholinesterase. Contact with a treated leaf or feeding on a treated plant results in insect paralysis followed by death (NACL Industries website) [27]. The toxic effect of Profen Super was studied on pollen grains of *Catharanthus roseus* (L.) G. Don. and *Clitoria ternatea* L. Inhibition of pollen germination and pollen tube elongation was used as parameter of toxicity. *C. roseus*, commonly called 'Madagascar Periwinkle', is a well known medicinal plant belonging to family Apocynaceae whereas *C. ternatea*, common name 'Butterfly pea', is a member of family Fabaceae that is used in traditional Ayurvedic medicine. Both plants have large sized pollen grains (51-100µm). *C. roseus* has spheroidal and tricolporate pollen grains (PalDat - Palynological Database A) [28] while pollen grains of *C. ternatea* are triangular and tricolpate (PalDat - Palynological Database B) [29].

Materials and Methods

Standardisation of germination medium for pollen culture

The flower buds of *C. roseus* and *C. ternatea* were plucked from the garden plants in the morning and placed under a table lamp in the laboratory to facilitate anthesis and anther dehiscence. Freshly dehisced anthers were gently tapped to collect the pollen grains. Pollen germination medium was standardised by raising hanging drop cultures of pollen grains and comparing their germinability in solutions of 20% sucrose, 30% sucrose or Brewbaker and Kwack medium (1964) [30] at ambient room temperature (30-33ºC). The best percent germination was observed in modified Brewbaker and Kwack medium (which contained 30% sucrose rather than the recommended 10%) after incubation for 60 minutes in the medium. Thereafter, all studies were carried out in the modified Brewbaker and Kwack medium.

Effects of different concentrations of Profen Super on pollen germination and pollen tube growth

A 35mL/16L aqueous stock solution of Profen Super E.C. was prepared as per the recommended dose by manufacturer (Rain Bio Tech Industries) [31]. Modified Brewbaker and Kwack germination medium was supplemented with suitable amount of the stock solution volume/volume, to obtain pollen germination medium containing 1:1, 1:2, 1:4 and 1:8 dilutions of Profen Super, respectively. Hanging drop cultures of pollen grains were set up in germination medium containing Profen Super as well as control (germination medium without the insecticide).

Hanging drop cultures of pollen grains

For raising hanging drop cultures, a drop of the germination medium was put in the centre of the lid of a cavity block and a thin layer of vaseline was applied along the edges of the lid. Some pollen grains were uniformly dispersed in the drop of medium using a needle. The drop was suspended in the cavity of the cavity block by carefully inverting the lid over it. After incubating pollen in the germination medium for 60 minutes, percent pollen germination was scored in three random non-overlapping microscopic fields. A pollen grain was considered to be germinated only when the length of pollen tube measured equal to or greater than its diameter. Pollen tube length was determined with an ocular micrometer. Those pollen grains whose pollen tube tips had burst were not scored. Average values for percent pollen germination and pollen tube length were taken into consideration for calculating percent pollen germination inhibition and percent pollen tube length inhibition in order to interpret the results.

Results

Good pollen germination for both the plant species was obtained in modified Brewbaker and Kwack medium. Higher germinability for pollen of *C. roseus* (81.38%) was observed in comparison to that of *C. ternatea* (71.70%). Germination of pollen in both the species was inhibited by all the tested dilutions of Profen Super and the inhibition increased with increase in concentration of the insecticide (Figures 1 and 2). Although % pollen germination was less in higher insecticide concentrations, hydration of pollen grains appeared to be normal. The values for average % pollen germination inhibition ranged from 11.48% (1:8 dilution) to 73.73% (1:1 dilution) for *C. roseus* while the values ranged from 56.08% (1:8 dilution) to 100% (1:1 dilution) for *C. ternatea* (Table 1; Figure 3). Thus, 1:1 dilution of Profen Super resulted in complete inhibition of pollen germination in *C. ternatea*. Inhibition of pollen germination was greater for *C. ternatea* compared to *C. roseus.* Both 1:8 and 1:4 dilution of the insecticide resulted in almost the same degree of pollen germination inhibition in *C. ternatea.*

Table 1: Effect of Profen Super on germination of *C. roseus* and *C. ternatea* pollen.

Dilution of Profen Super	*Catharanthus roseus*		*Clitoria ternatea*	
	Average % pollen germination	% Pollen germination inhibition	Average % pollen germination	% Pollen germinatcion inhibition
0 (Control)	81.38	0	71.70	0
1:8	72.04	11.48	31.49	56.08
1:4	66.03	18.86	31.01	56.75
1:2	26.23	67.77	13.46	81.23
1:1	21.38	73.73	0	100.00

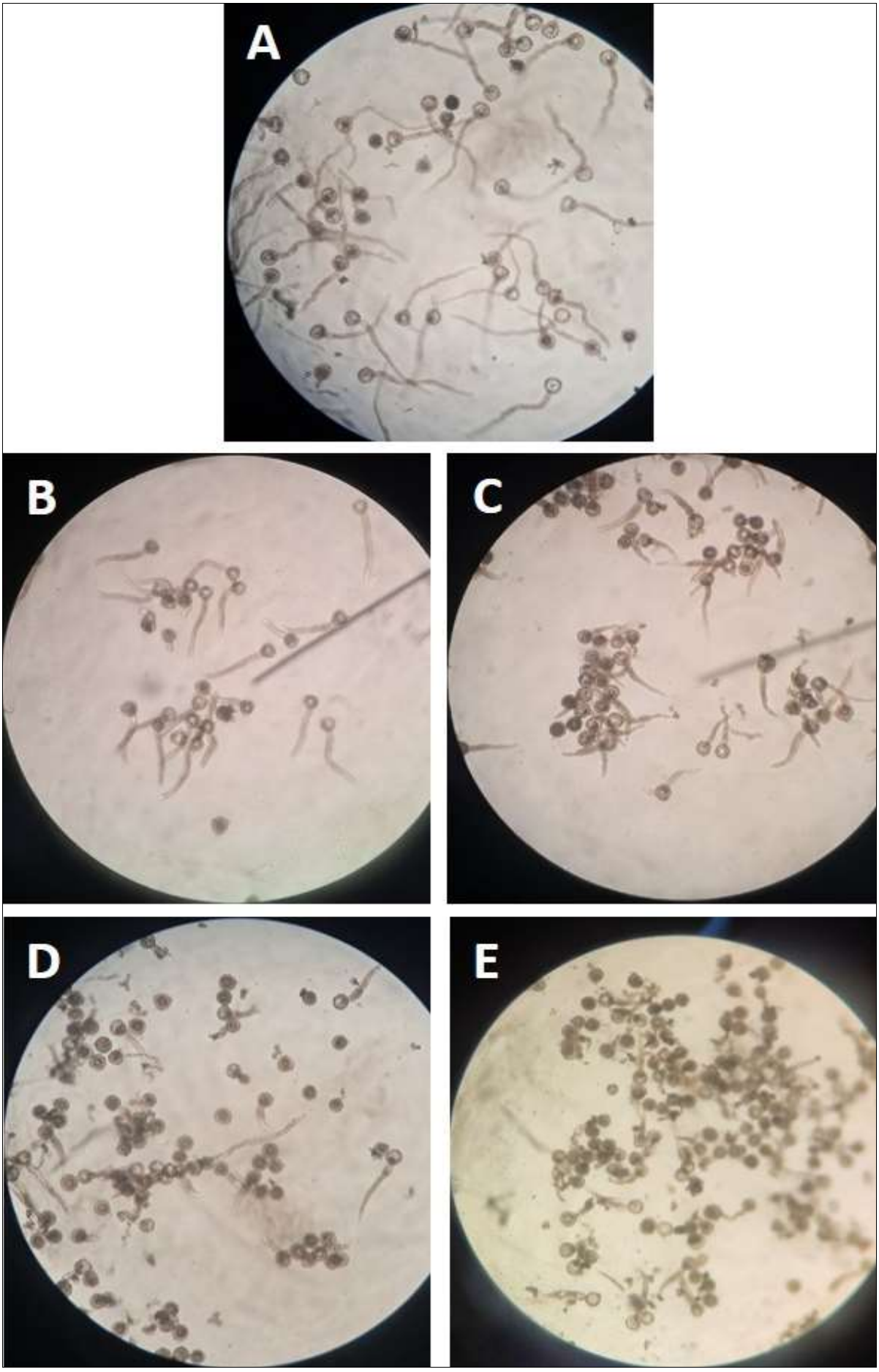

Fig 1: Micrographs showing effect of Profen Super on pollen germination and pollen tube length of *Catharanthus roseus* in 60 Minutes old pollen cultures. A. Control in modified Brewbaker and Kwack medium; B to E: In germination medium with 1:8, 1:4, 1:2 and 1:1 dilution of Profen Super, respectively.

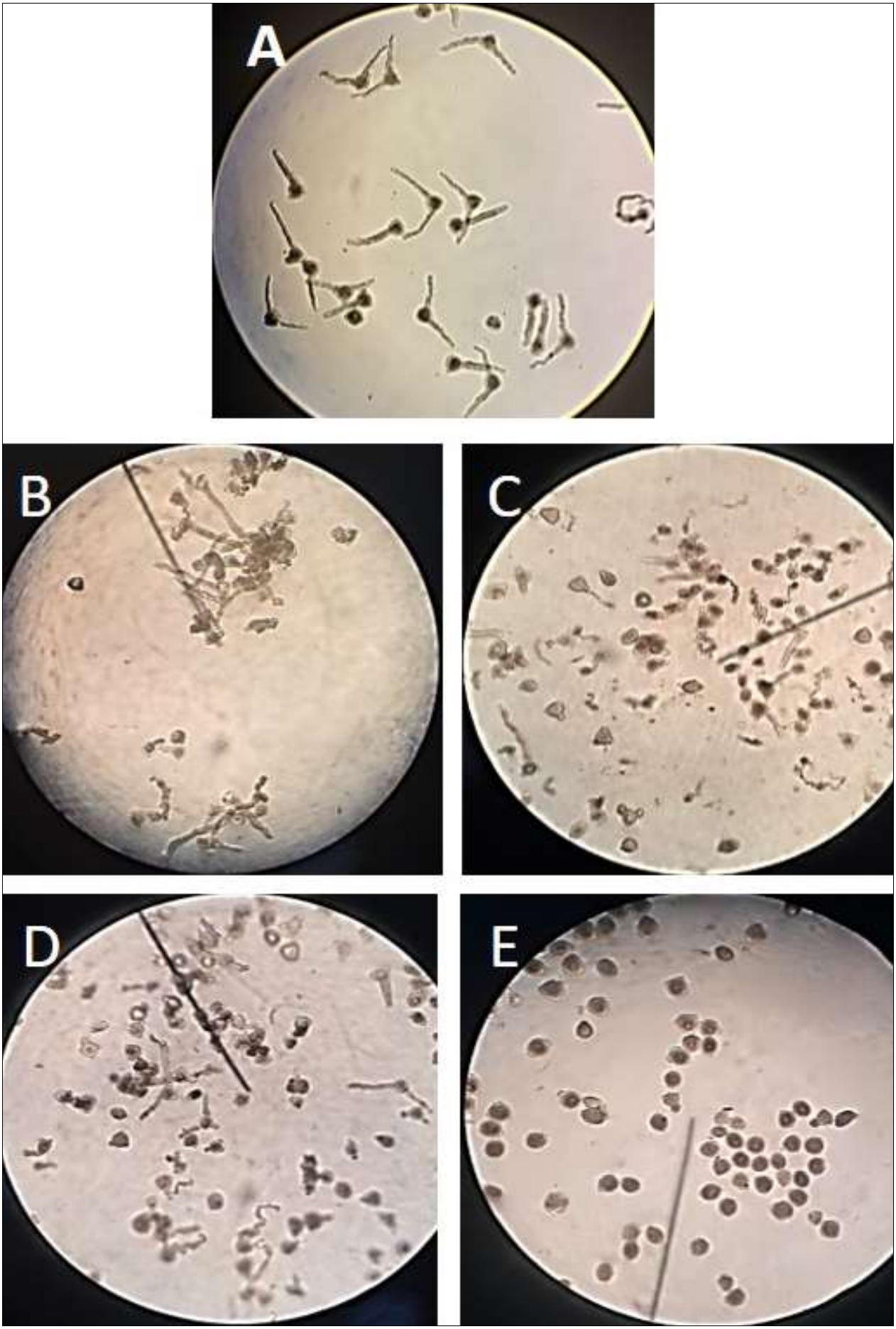

Fig 2: Micrographs showing effect of Profen Super on pollen germination and pollen tube length of *Clitoria ternatea* in 60 minutes Old pollen cultures. A. Control in modified Brewbaker and Kwack medium; B to E: In germination medium with 1:8, 1:4, 1:2 and 1:1 dilution of Profen Super, respectively.

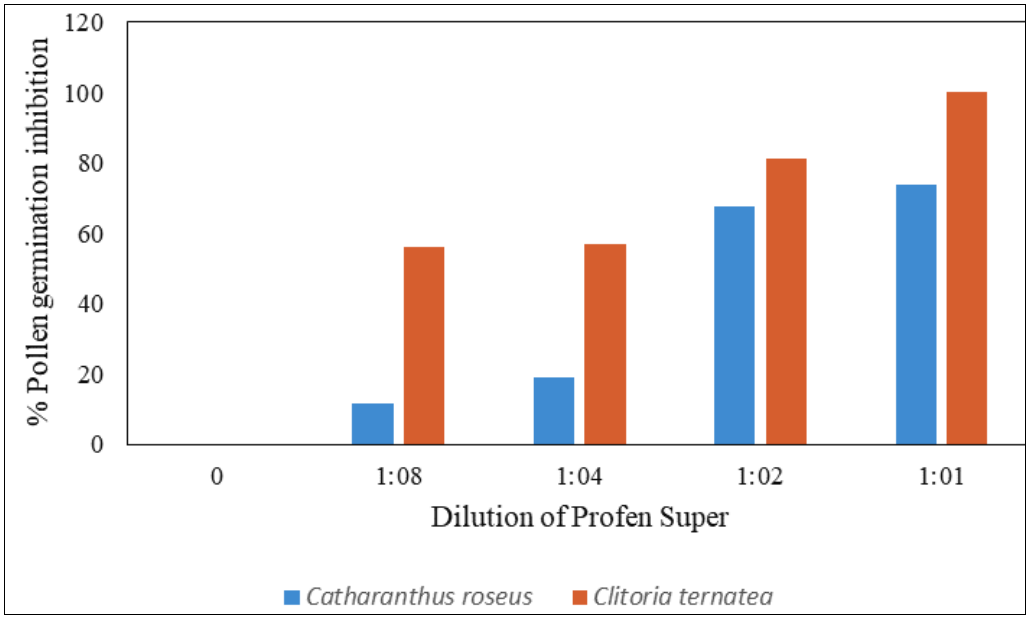

Fig 3: Pollen germination inhibition by Profen Super

In both *C. roseus* and *C. ternatea* some pollen grains produced more than one pollen tube as the pollen grains of the former are tricolporate and those of the latter are tricolpate. In the modified Brewbaker and Kwack medium (control) average pollen tube length was greater in *C. roseus* (325.5 µm) than *C. ternatea* (318.90 µm). In case of *C. ternatea* the average length of pollen tube decreased with increase in concentration of Profen Super. However, in *C. roseus*, though the pollen tube length appeared shorter in presence of the insecticide compared to control, the decrease Was not concentration dependent; both 1:8 and 1:4 dilution gave similar result (Table 2). The average % pollen tube length inhibition in *C. roseus* ranged from 34.77% (1:8 dilution) to 50.34% (1:1 dilution) whereas in *C. ternatea* the values ranged from 19.57% (1:8oncentration dependent dilution) to 100% (1:1 dilution) (Table 2; Figure 4). At higher concentrations of the insecticide (1:2 and 1:1 dilution) both species' pollen grains produced mostly papillae which did not elongate further (Figures 1 and 2).

Table 2: Effect of Profen Super on pollen tube length of germinated pollen of *C. roseus* and *C. ternatea*.

Dilution of Profen Super	*Catharanthus roseus*		*Clitoria ternatea*	
	Average pollen tube length (µm)	% Pollen tube length inhibition	Average pollen tube length (µm)	% Pollen tube length inhibition
0 (Control)	352.50	0	318.90	0
1:8	229.95	34.77	256.50	19.57
1:4	228.15	35.28	205.50	35.56
1:2	200.25	43.19	147.00	53.90
1:1	175.05	50.34	0	100

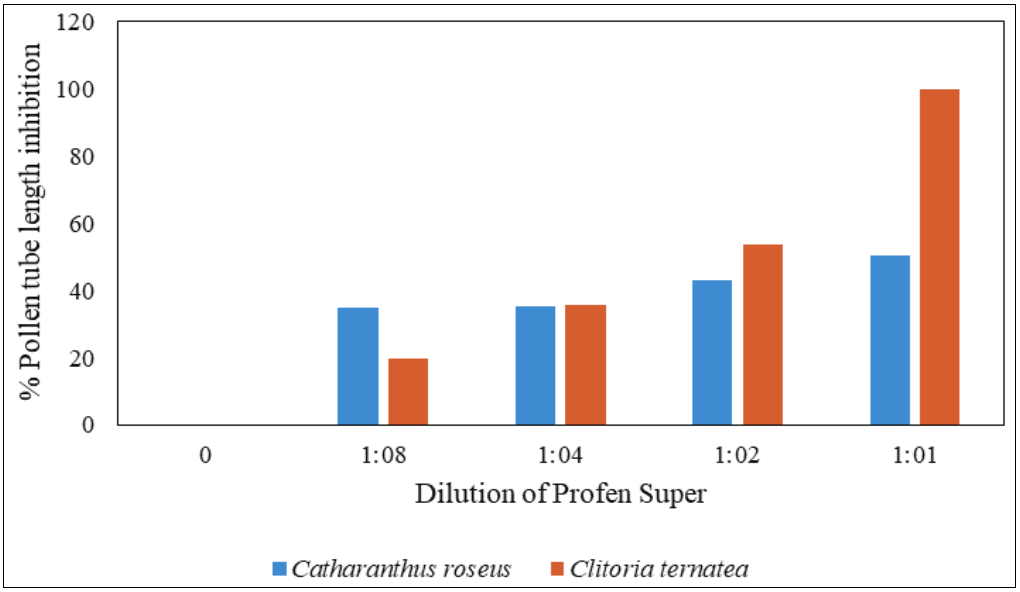

Fig 4: Pollen tube growth inhibition by Profen Super

Discussion

Pollen germination as well as pollen tube elongation were inhibited in both *C. roseus* and *C. ternatea* by Profen Super in the current study. Earlier investigations have illustrated that treatment with pesticide, especially fungicide, had an inhibitory effect on *in vitro* germination of pollen and growth of pollen tube in *Brassica campestris* (Pavlík and Jandurová, 2000) [25], *Brassica juncea* (Jain *et al*., 2000) [32], almond (Yi *et al*., 2003; Zarrabi and Imani, 2011) [33,34], peach and nectarine (Kargar and Imani, 2021) [35]. More specifically, *in vitro* pollen germination in tomato was reported to decrease with increase in concentration of Profex Super after the insecticide was sprayed on plants during flowering followed by collection of anthers for *in vitro* study (Meshram & Chaturvedi, 2017) [21]. Results of the present investigation corroborate the cytotoxicity of Profen Super demonstrated recently using the *Allium cepa* assay (Bahri *et al*. 2023) [36]. A suggested mechanism for the inhibitory effect is the interference by the fungicide with nutrient uptake or pollen metabolism (Holb, 2008) [37]. Inhibition of formation or fusion of Golgi vesicles, processes underlying the formation and elongation of pollen tube, could also be another possible mechanism (Subramanyan *et al*., 2023) [26]. The fact that exposure to the insecticide does not affect the hydration of pollen grains has been reported also in the study involving treatment of *Brassica campestris* with fungicide and it is proposed that the fungicide does not interfere with water uptake but may retard subsequent steps such as synthesis of pollen wall compounds or RNA synthesis (Pavlík and Jandurová, 2000) [25].

The pollen grains of a flower/plant are a heterogenous population consisting of segregated dominant and recessive genotype products of meiosis. It is likely that some stress tolerant genotypes exist in the plants because some pollen grains could germinate in presence of the chemical (Pavlík & Jandurová, 2000) [25]. In the present study, the inhibition of pollen germination occurred in a concentration dependent manner. Higher concentrations of Profen Super were highly inhibitory to pollen germination and pollen tube growth while lower concentrations of the insecticide allowed higher pollen germination and pollen tube growth. Thus, determination of the minimum inhibitory concentration of the toxic compound is necessary to allow its safe use on the plants for disease control.

Conclusion

This study reports the inhibition of pollen germination and pollen tube elongation *in vitro* in *Catharanthus roseus* and *Clitoria ternatea* on exposure to increasing concentration of the insecticide Profen Super. The results evidently point to the cytotoxic effect of this insecticide on pollen grains. Hence, spraying of the insecticide on the plants before fruit set should be avoided and its impact on fruit and seed set in plants must be investigated thoroughly before use.

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Conflict of interest

The authors declare that they have no conflict of interest.

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