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Impact of Azadirachtin on *in vitro* pollen germination of *Catharanthus roseus* (L.) G. Don. and *Clitoria ternatea* L

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Abstract
Several kinds of fungicides, insecticides and pesticides are used in crop fields to protect the plants from attack by different types of pathogens. Many of these synthetic chemicals have toxic effects on plants as demonstrated by experiments performed on germination of pollen grains under *in vitro* conditions. Pollen have been found to be a convenient system to assess the cytotoxic effects of chemicals. They can be easily cultured in a specific germination medium containing the chemical whose toxicity is to be assessed. In the present investigation, effect of a bioinsecticide Azadirachtin on pollen germination and growth of pollen tube of two medicinally important plant species, viz. *Clitoria ternatea* L. and *Catharanthus roseus* (L.) G. Don. under *in vitro* conditions has been studied. Pollen grains were cultured in germination media containing four dilutions of Azadirachtin i.e., 1:1, 1:2, 1:4 and 1:8. The observations clearly indicate a decrease in percent pollen germination and inhibition of pollen tube growth. Further studies should be carried out to determine the dose of bioinsecticide required for a particular crop and its time of application to combat its negative impact.

Keywords: Azadirachtin, bioinsecticide, pollen germination, *in vitro*

Introduction
In agriculture, use of insecticides, pesticides and fungicides is very common. But, continuous application of synthetic insecticides and pesticides over the years in agriculture has several detrimental effects on humans, plant growth and environment. It has resulted in accumulation of residues of pesticides in the environment. This in turn has led to various types of chronic illnesses (Bag, 2000) [2]. It has also caused environmental disturbances, leading to pest resistance and non-target organisms' toxicity. The harmful effects of synthetic pesticides have necessitated adoption of alternative means of control of insects/pests of crop plants. One such alternative method is use of bioinsecticides and biopesticides. Plant based insecticides and pesticides are ecofriendly and selective in their action. Among the biopesticides and bioinsecticides, neem plant-based products are the most potent and accepted widely (Chaudhary *et al*., 2017) [29]. Azadirachtin is the active compound in neem plant which is used for the production of biopesticides (Ashraf and Javaid, 2007) [9]. It has shown insect growth regulating activity for the insects' larval stages (Koul, 2007) [20].
Pollen grains can be considered as simple, inexpensive, reliable and useful systems for carrying out toxicological investigations (Kristen, 1997) [14]. There are several studies available in literature wherein effects of insecticides, pesticides, fungicides, herbicides and bioinsecticides on *in vitro* pollen germination have been studied (Gentile *et al*., 1971 [5]; 1973 [7]; 1978 [6]; Bilderback, 1981 [3]; Sutherland *et al*., 1984 [28] ; Jain *et al*., 2000 [11]; Nikolov *et al*., 2000 [19]; Kamble, 2005 [12]; Mehri *et al*., 2006 [15]; Salgare, 2006 [24]; Holb, 2008 [10]; Kargar and Imani, 2011 [13]; Meshram and Chaturvedi, 2017 [16]; Ghurde *et al*., 2023) [8]. These reports have clearly shown inhibition of *in vitro* pollen germination in the species investigated by the test chemicals. Germination of pollen grains under *in vitro* conditions and growth of pollen tube provide a convenient and sensitive method not only for studying the effects of toxic compounds but also for monitoring pollution (Shivanna & Rangaswamy, 1992 [27]; Shivanna, 2003) [26].
In the current investigation, effects of bioinsecticide Azadirachtin (neem oil) were studied on percent pollen germination and pollen tube length of two important plant species, viz. *Clitoria ternatea* L. and *Catharanthus roseus* (L.) G. Don. *C. ternatea* is commonly known as 'Butterfly pea'. It is a member of Fabaceae family. It is used in traditional Ayurvedic medicine for treating many diseases. It has several bioactive compounds including steroids, flavonol glycosides, triterpenoids, and anthocyanins. The plant extract possesses many pharmacological activities (Mukherjee *et al*., 2008) [17]. *C. roseus* belongs to family Apocynaceae and is commonly known as Madagascar periwinkle. It plays a significant role in traditional and herbal medicine for treatment of several diseases and is also an important species in horticulture (Nejat *et al*., 2015) [18]. The therapeutic potential of the plant has been attributed to the presence of several phytochemicals in it. Many alkaloids and phenolics have been isolated and identified from it that possess various biological activities (Pham *et al*., 2020) [20]. *C. roseus* has spheroidal and tricolporate pollen grains (PalDat - Palynological Database A) whereas the pollen grains of *C. ternatea* are triangular and tricolpate (PalDat - Palynological Database B). The pollen grains of both flowers are monads and large in size (51-100µm).

Materials and methods
Standardisation of *in vitro* pollen germination medium
The flower buds of *C. ternatea* and *C. roseus* were collected from the garden in the morning. To select the optimum medium for pollen germination under *in vitro* conditions, pollen grains of these two plants were germinated in 20%

sucrose solution, 30% sucrose solution and Brewbaker and Kwack medium (1964) [4] at room temperature (30-33ºC) by hanging drop culture technique. The highest percent pollen germination was observed in modified Brewbaker and Kwack medium containing 30% sucrose after one hour of incubation in the medium. This optimum medium was used for performing all the experiments.

Preparation of different dilutions of Azadirachtin

A 0.15% aqueous stock solution of commercially available Azadirachtin oil (Neem Kernel Extract 60.00% W/W in solvent methanol containing min 0.15% Azadirachtin Emulsifer 5.00% W/W, Polyethene ether and treated neem oil 35.00% W/W manufactured by Ved Vyas Agro and Chemicals) was prepared. Requisite amount of the stock solution was added to the modified Brewbaker and Kwack germination medium to obtain 1:1, 1:2, 1:4 and 1:8 dilutions of Azadirachtin (V/V). Hanging drop cultures of pollen grains were raised in germination medium containing Azadirachtin solution as well as control (germination medium without bioinsecticide).

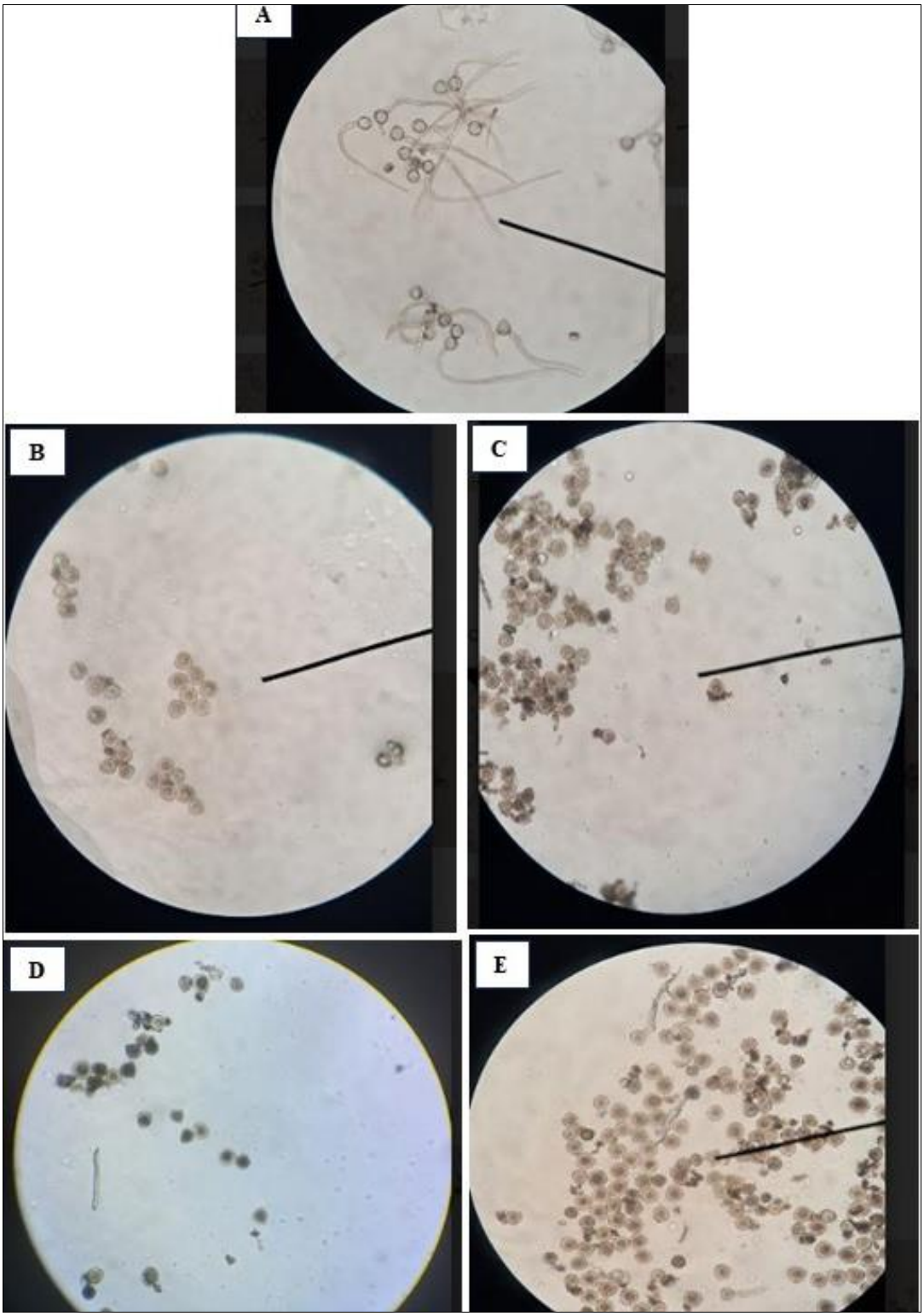

Fig 1: Photographs showing effect of Azadirachtin on pollen germination and length of pollen tube of *Catharanthus roseus* after one hour of incubation. A. Control in modified Brewbaker and Kwack medium; B to E: In 1:1, 1:2, 1:4 and 1:8 dilution of Azadirachtin, respectively.

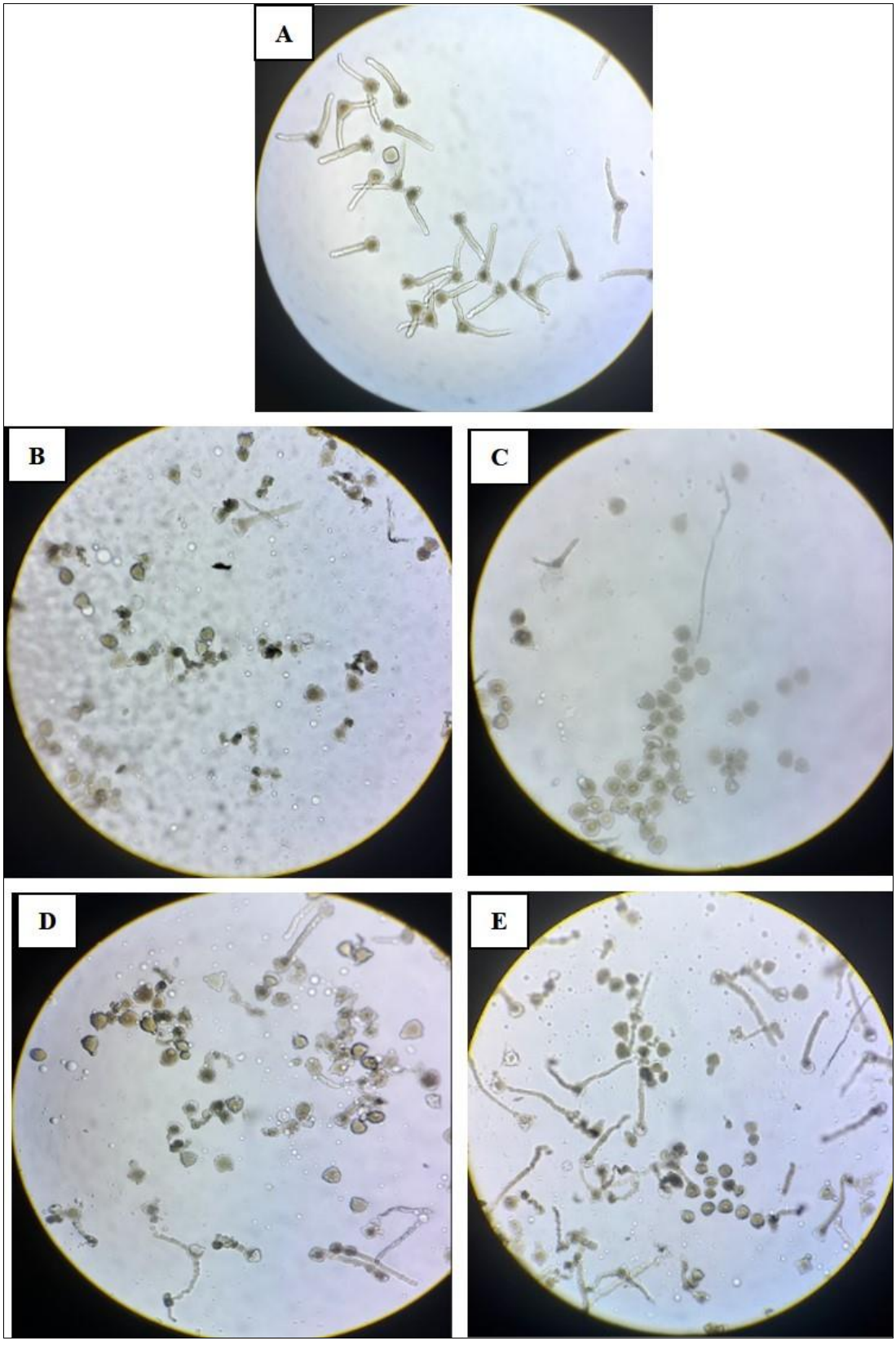

Fig 2: Photographs showing effect of Azadirachtin on pollen germination and length of pollen tube of *Clitoria ternatea* after one hour of incubation. A. Control in modified Brewbaker and Kwack medium; B to E: In 1:1, 1:2, 1:4 and 1:8 dilution of Azadirachtin, respectively.

Hanging drop cultures

To raise hanging drop cultures, a drop of the germination medium was placed on the cover glass of a cavity block and Vaseline was applied on its edges. On the drop of medium, a few pollen grains were dusted and mixed with the help of a needle to obtain a homogenous suspension. After this, the cover glass with the culture drop was inverted and placed over the cavity of the cavity block carefully and its edges pressed gently to seal the cavity taking care that the drop did not spread but hung over the cavity of cavity block. After incubation for one hour in the germination medium, total number of pollen grains and number of germinated pollen grains were counted in three random non-overlapping

Microscopic fields to calculate percent pollen germination. Only those pollen grains were considered as germinated whose pollen tube length was equal to or more than their diameter. To measure the length of the pollen tubes, an ocular Micrometer was used. All cultures were raised thrice and their average values were taken for result analysis.

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

Dilution of Azadirachtin	*Catharanthus roseus*		*Clitoria ternatea*	
	Average % pollen germination	% Pollen germination inhibition	Average % pollen germination	% Pollen germination inhibition
0 (Control)	39.16	0	54.12	0
1:8	0.79	97.98	27.27	49.61
1:4	0	0	21.42	60.42
1:2	0	0	2.22	95.89
1:1	0	0	2.50	95.38

Table 2: Effect of Azadirachtin on germination of *C. roseus* and *C. ternatea* pollen tube length.

Dilution of Azadirachtin	*Catharanthus roseus*		*Clitoria ternatea*	
	Average pollen tube length (µm)	% Pollen tube length inhibition	Average pollen tube length (µm)	% Pollen tube length inhibition
0 (Control)	277.5	0	315.00	0
1:8	15.0	94.59	150.00	52.38
1:4	0	0	123.75	60.71
1:2	0	0	75.00	76.19
1:1	0	0	80.00	74.60

Results

The experimental results demonstrate that Azadirachtin inhibits pollen germination as well as reduces pollen tube length under *in vitro* conditions in both the plant systems namely, *C. roseus* and *C. ternatea* (Figures 1 and 2). In case of *C. roseus*, an average of 39.16% pollen germination was obtained as compared to 0.79% at 1:8 dilution of Azadirachtin. Surprisingly, none of the pollen germinated in 1:1, 1:2 and 1:4 dilutions of Azadirachtin. Pollen tube length was very small (15.0µm) as compared to the control (277.5µm). In contrast to this, pollen germination was observed in control as well as all the dilutions of Azadirachtin tested in *C. ternatea*. The highest percentage Of pollen germination in *C. ternatea* was observed in control (54.12%). As compared to control the percent pollen germination was quite low in all the dilutions of Azadirachtin tested (Table 1, Figure 3). With respect to length of pollen tube in *C. ternatea*, the highest pollen tube length was obtained in control followed by 1:8 and 1:4 dilutions of Azadirachtin, respectively (Table 2, Figure 4). The average values for % pollen germination inhibition ranged from 49.61 at 1:8 dilution to 95.89 at 1:2 dilution, respectively. Percent pollen tube length inhibition obtained at 1:1 (74.60%) and 1:2 (76.19%) dilutions of Azadirachtin was almost the same while for the remaining two dilutions it was much higher.

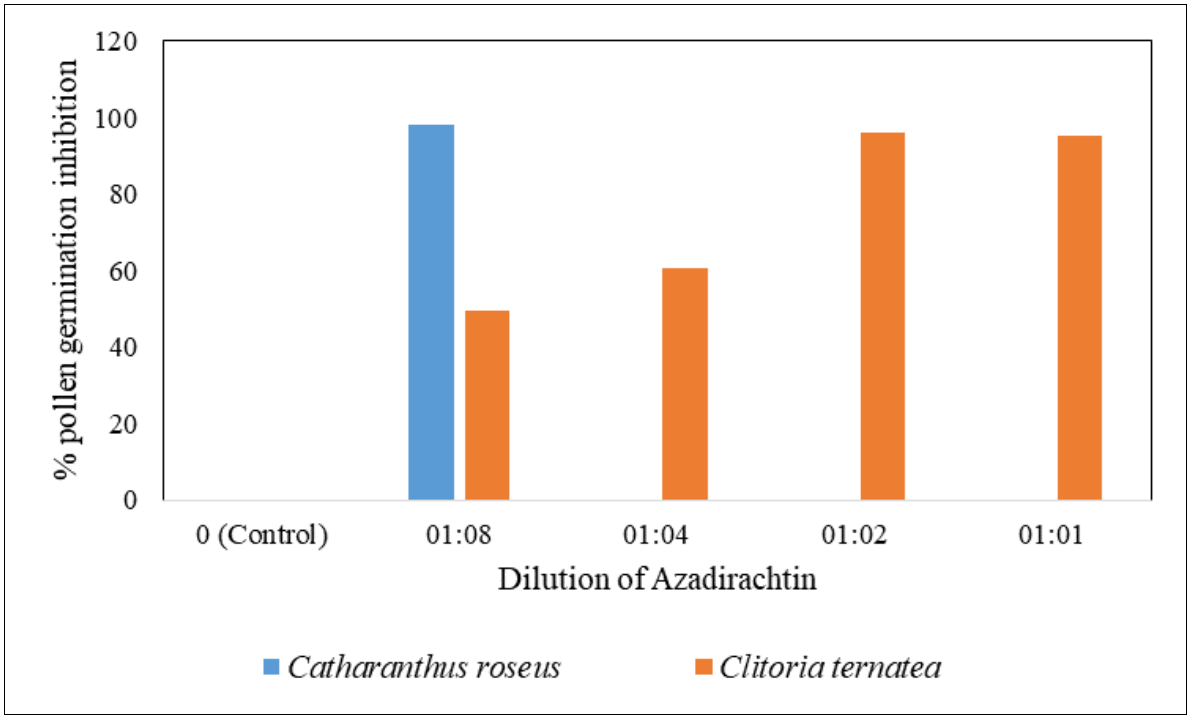

Fig 3: Inhibition of pollen germination by Azadirachtin

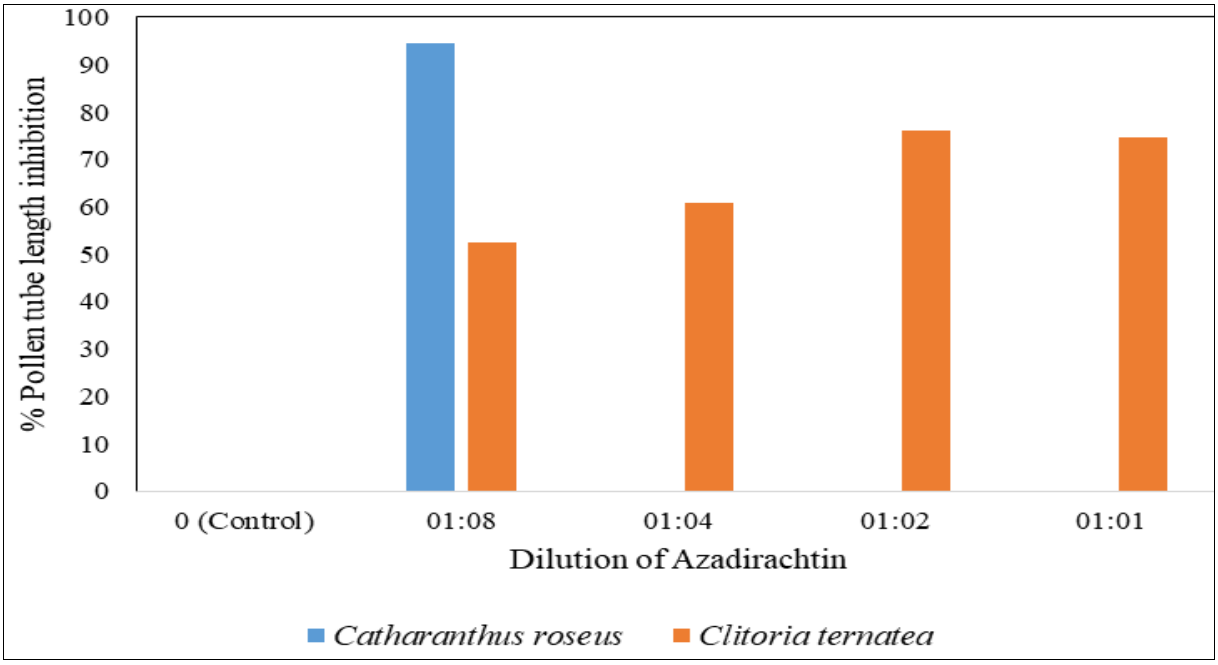

Fig 4: Inhibition of pollen tube length by Azadirachtin

Discussion

Our study indicates that all the dilutions of Azadirachtin tested resulted in inhibition of *in vitro* pollen germination as well as pollen tube growth. It was observed that higher concentrations of the bioinsecticide resulted in more growth inhibition as compared to lower concentrations tested. A study carried out in mung bean plants by Sarawaneeyaruk *et al*. in 2015 [25] also found that use of Azadirachtin and neem extract reduced the number of root nodules. Further, Azadirachtin was found to decrease soil and rhizosphere microorganisms' population. In another study, when effects of organic insecticides, Kingbo and Azdar 10 EC were tested on mitotic chromosomes in root tip cells of *Allium cepa*, an increase in frequency of chromosomal aberration and decrease in mitotic index was reported (Al-Ahmadi, 2013) [1]. Since the results show that Azadirachtin is extremely inhibitory for *C. roseus* pollen while in *C. ternatea*, it is less inhibitory, the impact of bioinsecticide appears to be species specific. Hence, it is necessary to check its effect on the plant before treating it with the insecticide.

Conclusion

The bioinsecticide Azadirachtin caused inhibition of pollen germination *in vitro* and also elongation of pollen tube in *Catharanthus roseus* and *Clitoria ternatea*. The present investigation indicates that the biopesticides and bioinsecticides should be tested for their effect on pollen grain germination before their application on crops.

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Conflict of interest

The authors declare that they have no conflict of interest.

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