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Assessing the Morphological Variations on the Pollen Grains of *Solanum Betaceum* caused by Chemical, Biological and Ecological Pesticides

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Abstract

Solanum betaceum is a crop of great economic importance in Ecuador. Unfortunately, it is susceptible to diverse pathologies caused by bacteria, fungi, insects and nematodes. Several pesticides are currently used to prevent the plagues but their intensive and/or incorrect use can affect the development of the plants as well as the local environment. The aim of this paper is to investigate the morphological changes on both the internal and external structure of the of *Solanum betaceum* pollen grains caused by three types of pesticides: chemical, biological and ecological. By using Transmission and Scanning Electron Microscopy, we have studied over 140 pollen grains exposed to these different pesticides. The results demonstrate that the pesticides influence the morphology and structure of the pollen grains, thus probably affecting the plant reproductive process.

Keywords: Pesticide; TEM; Pollen; Tomato tree

Introduction

The tamarillo (*Solanum betaceum* Cav. syn Cyphomandra betacea Sendt.), also known by its popular name as tree tomato, is native to the Andes and has been traditionally cultivated in Ecuador, Peru, Bolivia and Colombia [1]. The tree tomato is susceptible to several diseases caused by bacteria, fungi, nematodes and insects, which generate damages on the leaves, roots, flowers and pollen. Pesticide application is the common agricultural solution to eliminate such pathogens. Obviously, the wrong use of pesticides has several effects on plants, animals and people and can negatively impact the local ecosystems [2,3]. Both *in vitro* and *in vivo* experiments showed that the use of pesticides causes inhibition of pollen germination in several crops leading to plant infertility [4]. It has also been shown that in red tomato, *Lycopersicum esculentum*, the use of chlorotalonil fungicide prevents pollen germination and produces a negative effect on fruit development [5]. Moreover, it was demonstrated [6] that the *in vitro* exposure to the chemical pesticide chlorotalonil on melon, *Cucumis melo L*, reduces the percentage of germinated pollen and generates an impact in the length of the germ-tube elongation. The tomato tree is an excellent source of vitamins A and C, calcium, potassium, phosphorus, sodium and magnesium, however, there is a lack of information about the effects of the pesticides used for this crop [7]. The main goal of this work is to investigate and compare the internal and external changes on morphological structures of *Solanum betaceum* pollen induced by three types of pesticides: chemical, ecological and biological.

Materials and Methods

Sample collection

Flowers of tomato tree were randomly collected from the crops of Selva Alegre, near the campus of Universidad de las Fuerzas Armadas ESPE, in Sangolquí, Pichincha, Ecuador. Subsequently, the pollen grains were manually extracted in the Centro de Nanociencia y Nanotechnology located in the same University.

Pesticides exposition

Six different pesticides were used, two for each type: chemical, ecological and biological. The methodology and procedures used for the application of the pesticides were provided by local farmers (Table 1).

Name	Type	Pesticide Compound	M/E
A	CHEMICAL	Chlorothalonil	2.5 – 3.0 cc/l
B	CHEMICAL	Pyraclostrobin + epoxiconazole	0.5 l/ha
C	ECOLOGICAL	Extracto Carboxamida	300 g/ha
D	ECOLOGICAL	Hydroxymethyl Alkyl N dimethyl	300 g/ha
E	BIOLOGICAL	Metarhizium anisopliae	1 g/l
F	BIOLOGICAL	Lecanicillium lecanii	1.25 cc/l

Table 1: Concentration of the six pesticides.

All extracted pollens, randomly chosen, were located into a germination medium composed of sucrose and boric acid. There were exposed to a 1 ml pesticide dilution during 6 hours at 22°C. Additionally, two germinating pollen samples were used as control in the same conditions.

Transmission electron microscopy

Specimens were fixed in a 2% glutaraldehyde solution for 16 hours at 4°C. After washing in a phosphate buffer, 5 min - twice, the samples were post-fixed in a 1% osmium tetroxide solution for 1 hour at 4°C. Fixed samples were then washed with distilled water during 5 min - twice, and dehydrated in a graded ethanol series: 30%, 50%, 70%, 80% and 90%, for one hour each. The last dehydration was carried out with a 99.9% ethanol solution overnight. An ethanol and acetonitrile in a 1:1 ratio and then a 100% acetonitrile were used as a transition fluid solution, 1 hour each. Infiltration was done with EPON resin and acetonitrile in the following ratios: 1:2; 1:1; 2:1, each for 12 hours. This was followed by an overnight infiltration in a 100% EPON resin. Samples embedding were carried out for 72 hours at 60°C. Resin embedded blocks were sliced with glass knives using an ultra-microtome PowerTome© from RMC set at a 1.5 mm/s speed with a section thickness of 100 nm. Slices were transferred to moist glass slides. TEM observation sections were collected on gold-coated meshes that were previously heated. 1% uranyl acetate and 2% lead citrate were used as double positive staining. Electron micrographs were taken on a FEI TECNAI G2 SPIRIT operating at 80 kV.

Statistical analysis

Statistical significance of the morphological changes caused by the different pesticides on the pollen grain was calculated using a binomial logistic regression. H0 hypothesis was defined as pesticides exposition affects significantly the pollen morphology. For each pesticide, 20 randomly chosen pollen grains were used for this study.

Results and Discussion

Control samples

The control sample was fresh pollen unexposed to any pesticide, and was immersed directly in the germination medium. This control corresponds to a natural tomato pollen state. The external morphological features of pollen show three protective layers: ectexine, endexine and intine [8]. Figure 1a shows a pollen grain size of about 22 µm with a radial symmetry and tricolporate structure, which corresponds to the classical structure of Solanaceae family [9]. The micrographs obtained from ultrathin cut (Figure 1b) help to distinguish the internal structure of pollen where two layers, exine (EX) thicker than intine (IN), cover the pollen grain. One can also observe the presence of abundant cytoplasm with the vegetative nucleus (VN) located within the central region of the structure close to an aperture; and the generative nucleus (GN) located next to the intine layer [10].

The second control corresponds to a germinated pollen grain exposed to a germination medium during six hours (Figure 1c). The SEM image reveals the formation of a pollen tube of around 15 µm length. On the other hand, the TEM images (Figure 1d) show the presence of the two nuclei and a complex cellular structure, in which one can easily distinguish the plastids filled with starch granules.

Chemical pesticides

Two chemical pesticides labelled "A" and "B" were applied. As it can be observed in Figure 2, the pesticide "A" induces significant variations in the morphology: pollen grains are completely deformed and do not show recognizable internal structures in 80% of the samples. In Figure 2a one can identify the pollen tube formation, but there is no formation of internal nuclei. Figure 2b depicts what is believed to be the vegetative nucleus (VN) but the surrounding material lacks of shapes of known structures at all. The second chemical pesticide labelled "B" induces the same abnormalities as the "A" pesticide, but for this case the percentage increases up to 85% of the samples. In most of the cases, a strong variation in the pollen grain morphology is observed making it impossible to distinguished internal organelles in the cytoplasm and the germinated pollen tube, as depicted in Figure 2c. Some pollen grains show a pollen tube formation (Figure 2c and d), but such grains do not have inner nuclei or organelles. Finally, in the studied sections with presence of germinating and growing nuclei, it has never been identified the presence of a pollen tube.

Ecological pesticides

The two ecological pesticides applied for the study are labelled "C" and "D". The first pesticide, "C", induces a tricolporate structure without protrusions or apertures (Figure 3a and b) and shows a different location for the vegetative and generative nuclei. However, it is still possible to distinguish internal organelles as generative cells within its nuclei and the presence of more than one pollen tube (Figure 3b). In 35% of the cases morphological alterations have been clearly evidenced. The second ecological pesticide labelled "D" induces elongated morphologies. Some pollen grains appear to have two pollen tubes in formation, as depicted in Figures 3c and d. The ecological pesticides induce the presence of cells without a defined generative nucleus and variations in the nuclei size. A lack of shape of the vegetative nucleus is evidenced in 65% of the studied cases.

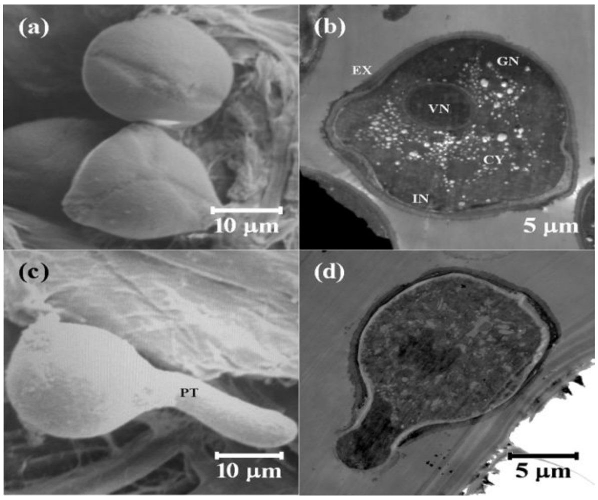

Figure 1: (a) Polar view of a non-germinated pollen, with no pesticide effects, (b) A 100 nm thick pollen section. EX: exine, IN: intine, VN: vegetative nucleus, GN: generative nucleus, CY: cytoplasm, (c) A germinated pollen grain showing the pollen tube (PT) structure growing in one of the apertures, (d) Section of germinated pollen showing its nuclei and other internal structures.

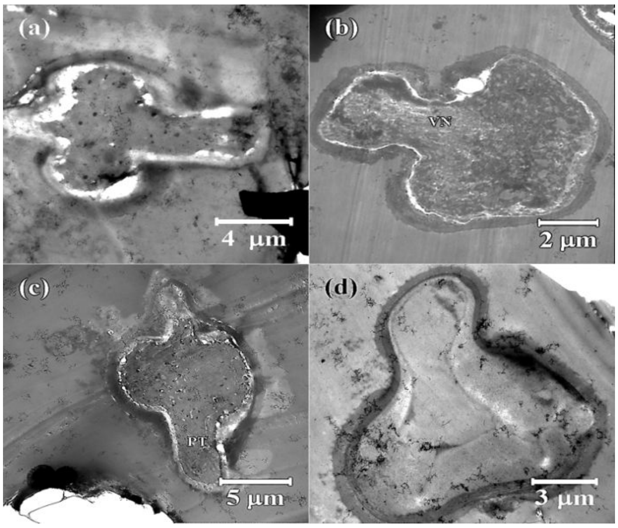

Figure 2: 100 nm sections of *Solanum betaceum* pollen exposed to chemical pesticides "A" (a-b) and "B" (c-d). (a) Deformation in pollen morphology, (b) Example of pollen tube with presence of a vegetative nucleus (VN), (c) Pollen tube formation and morphology, (d) Pollen morphology deformation similar to (a).

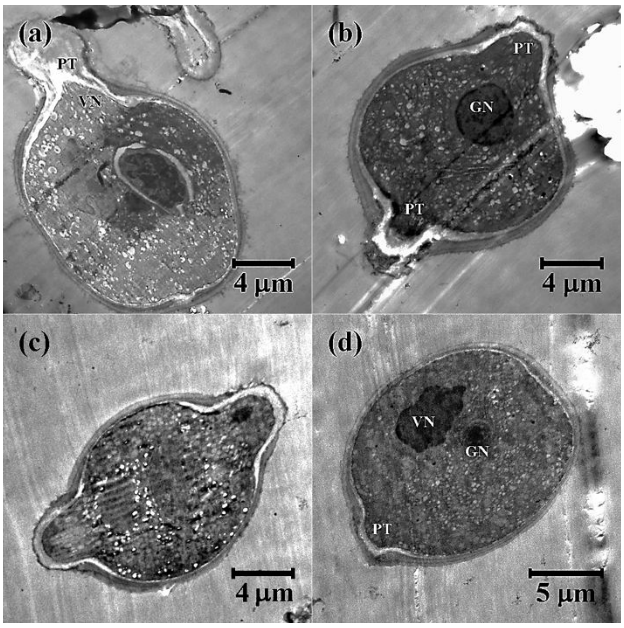

Figure 3: *Solanum betaceum* pollen treated with organic pesticide labelled "C" (a-b) and "D" (c-d). (a) The vegetative nucleus (VN) is located close to the pollen tube, (b) Formation of two pollen tubes, (c) No evidence of tricolporated structure but there are two pollen tubes in formation, (d) Morphology with a single aperture (PT) showing a large vegetative nucleus.

Biological pesticides

Two biological pesticides labelled "E" and "F" were applied. The samples exposed to the pesticide "E" show a slight variation in the morphology of pollen grains: these are usually spherical and sometimes do not maintain the tricolporate structure (Figure 4a). Other pollen grains remain with the characteristic shape of the family Solanaceae (Figure 4b). Regarding the internal structure, all pollen grains have large generative nucleus (GN) and vegetative nucleus (VN) near the apertures. In almost all the TEM micrographs it is possible to distinguish the exine surrounding the pollen grain, the intine outlining the cytoplasm and the formation of a pollen tube. The samples treated with biological pesticide "F" show, in 30% of the studied cases, three types of changes: an irregular variation of tricolporate shape (Figure 4c and d), the formation of two pollen tubes, and also an abnormally high quantity of plastids filled with starch throughout the cytoplasm. As shown in Table 2, all pesticides generate morphological damages.

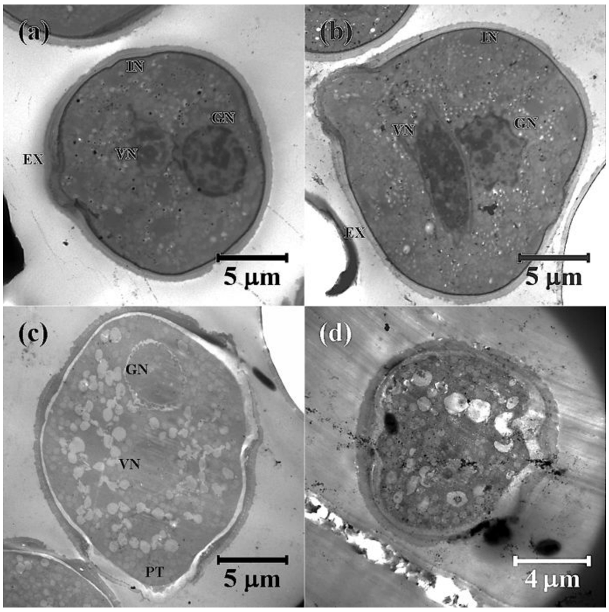

Figure 4: *Solanum betaceum* pollen grain treated with biological pesticide "E" (a-b) and "F" (c-d). (a) Typical observed spherical form, (b) Pollen grain maintaining the shape of the Solanaceae family, (c) Formation of a pollen tube (PT) and a larger germinative nucleus, (d) Variation of the characteristic shape structure of Solanaceae family and evidence of abundant plastids.

Name	% of pollen observed with morphological defects	Name	% of pollen observed with morphological defects
Control	25		
A	80	D	65
B	85	E	20
C	35	F	30

Table 2: Percentage of morphological defects observed in pollen for each pesticide.

In the specific case of the chemical pesticides, a clear difference is observed with the control sample. In most of the TEM pictures, the organelles and the tricolporate structure are no longer observed, the exine is lost and the intine layer is no more recognizable. For "A" chemical type, chlorothalonil blocks the transformation of alternative structure of glutathione and reduces enzyme activities [11], which is an antioxidant enzyme that plays an important function in the cellular respiration process. Moreover, it inhibits the action of the oxidase and NADPH in the process of glycolysis, and also inhibits the glyceraldehyde 3-phosphate dehydrogenase. This could explain the observed effects on pollen structure. The compounds of the chemical pesticide "B" are pyraclostrobin and epoxiconazole. Both make a systemic fungicide used to control foliar diseases. In most part of the samples, a lack of organelles is observed, due to the fact that epoxiconazole inhibits the biosynthesis of phytosterol, a constituent of the cell membrane of fungi [12]. Pyraclostrobin acts as an inhibitor of electron transport in the mitochondria of fungi cells by inhibiting the formation of ATP [13]. The combined action of pyraclostrobin and epoxiconazole produces free radicals and affects ubiquinol oxidase enzyme cytochrome bc1 complex (Elskus 2012). The produced radicals alter mitochondrial and cytoplasmic membranes inhibiting future growth of the fungus. For these reasons strong morphological changes in the ultrastructure of pollen are observed. For ecological pesticide, some differences are observed in nuclei location. "C" compound is a carboxamide extract, and is applied on crops for inhibiting the growth of fungi. Its action inhibits the enzyme succinate dehydrogenase ubiquinone complex II and disrupts the mitochondrial electron transport in the mitochondrial respiratory chain. Consequently, the fungus remains without energy and is eliminated [14]. This seems also to have a consequence in the internal structure of the pollen, but only in a few cases: 35%. The other ecological pesticide "D" is made by Hydroxymethyl Alkyl N Dimethyl. It is used as a contact fungicide and bactericide of systemic action. It initiates a competition for oxygen with the phito-pathogen. This harmful effect on pollen morphology is less than the chemical pesticides, but still high: 65%. In recent years the use of fungus *Metarhizium anisopliae*, "E" case, has been extensively reported as a biological pesticide, mainly due to the conidia production on a large scale [15]. It is a known insect pathogenic fungus, which infects a wide range of insects. In the present study, we show that this biological pesticide does not cause major deformation of the internal pollen grains structures. This is mainly due to the fact that it only affects insects, through the dtxS1 gene [15]. The other entomopathogenic fungus, *Lecanicillium lecanii*, is labelled "F" in this study, also known as mycoparasites of species of pathogenic plant, biotrophic powdery mildew, rust fungi on various vegetable, fruit and ornamental crops [16], and as pathogens of plant parasitic nematode [17]. These fungi have a suppressive effect that may be due to the inhibitory action on mitochondrial respiration by affecting the NADH-cytochrome C- reductase and complex- I of insect mitochondria [18], leading to the death of the host by physiological starvation. Ownley et al. [17] suggest that the endophyte infection might result in positive effects such as an enhancement of the plant growth. Our study only confirms that Lecanicillium does not affect the development of pollen grain, as no significant difference is observed with the control. Finally, in order to verify a statistical difference between group one pesticides and group two no pesticide, the odd ratio of the two groups is calculated: 3.2. This clearly confirms the H0 hypothesis that pesticides exposition affects significantly the pollen morphology.

Conclusion

We have evaluated the impact generated by the tree pesticides` groups on the internal and external structure of tomato tree *Solanum betaceum* pollen. The most harmful pesticides' group was the chemical one, generating morphology damages in the 80% of pollen grains analyzed. It was followed by the ecological 50% and the biological 10% pesticides. As one might expect, this could significantly affect the sexual reproduction of the plant. This hypothesis has to be corroborated and will open new avenues for studying the impact of pesticides on fruit production.

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