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Calcium Oxalate and Other Cladode Features in *Opuntia ficus-indica* Resistant Cultivars to *Dactylopius coccus* Costa

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Abstract

Multipurpose cactus pear plant with great potential as a source of food and livestock faced a threat from *Dactylopius* spp in different countries. Specifically, *D. coccus* is an important pest damaging significant areas in Tigray-Ethiopia. Using pest-resistant cultivars is an important element of an integrated pest management strategy, and studying the mechanisms of resistance is vital. It can be chemical or physical such as oxalate crystals and other cladode characteristics. Cladode features of six cultivars (three *O. ficus-indica*, two *O. cochenillifera*, and one *O. robusta*) were examined for resistance to *D. coccus* in a completely randomized design (CRD) with three replications. 'Rojo Pelón' (*O. ficus-indica*), 'Robusta' (*O. robusta*), and 'Bioplástico' (*O. cochenillifera*) are resistant cultivars; and 'Atlixco' and 'Chicomostoc' (*O. ficus-indica*) and 'Nopalea' (*O. cochenillifera*) are susceptible. Cultivars showed a significant difference in cladode weight in g, and cladode length, cladode width, and cladode thickness in cm, where cladode thickness was higher in 'Rojo Pelón' followed by 'Robusta'. Calcium oxalates number per mm was higher in 'Bioplástico' (20.7 ± 2.08) followed by 'Robusta' (18.9 ± 2.31) and 'Rojo Pelón' (15.9 ± 0.34); and similarly, epidermis thickness found higher in 'Bioplástico' (0.21 ± 0.032) and 'Robusta' (0.19 ± 0.014), but similar with 'Rojo Pelón' (0.18 ± 0.026). However, cuticle thickness didn't show a difference among cultivars. Cladode thickness, calcium oxalate number, and epidermis thickness had positive correlations with resistance. These results demonstrate that calcium oxalate number and epidermis thickness might have a positive role in

D. coccus resistance in *O. ficus-indica*. This feeding-barring role and the insect-plant interaction need to be studied.

Keywords

Cactus Pear, Resistance, Druses, Epidermis Thickness

1. Introduction

Cactus pear (*Opuntia ficus-indica)* has many uses and huge potential mainly as human food and forage, its advantages include good biomass yield, it can grow on marginal land, being year-round evergreen, tolerant to drought, and crop during difficult times [1] [2]. It also gives better yield in suitable lands [3]. However, it is threatened by *Dactylopius* species on different continents. *D. coccus* damaged 75,000 ha in Tigray, Ethiopia [4] [5] and *D. opuntiae* is a devastating pest in Brazil and the Mediterranean region [6] [7] [8].

The development and use of resistant varieties are advantageous for the economy, ecology, and environment [9]. Understanding the levels and mechanisms of resistance is paramount for integrated pest management strategies [10] [11]. There are some resistant cultivars of *O. ficus-indica* to *D. coccus*. [12] and [13] reported differences in yield among *Opuntia* varieties in Mexico and discussed that resistance may occur. [14] identified the cultivar 'Rojo Pelón' (*O. ficus-indica*) resistant to this insect.

Mechanisms of resistance could be due to phytochemicals [11] or mechanical barriers such as calcium oxalate crystals and histological structures [15]. Higher concentration of these crystals may make it difficult for nymphs to insert their stylets and settle on the cladodes [16].

Naturally formed mineral crystals in plants can serve as a very effective insect defense [16] [17]. Calcium oxalate can be a physical barrier against chewing insects by an abrasive effect that blunts insects' mandibles and may act as an anti-nutritive defense by decreasing the efficiency with which ingested food is digested [18] [19]; so, it may deter the feeding of beetles [20]. Soluble oxalate or oxalic acid protects plants from herbivory by sucking insects [21] [22] [23]. However, the protective role of calcium oxalates can be observed in certain plants and need trial confirmation [24]. The presence of calcium crystals in different *Opuntia* spp was reported [25] [26] and the accumulation level varies depending on cultivars, growth stage, and other agronomic conditions [27] [28].

The cactus pear stem has a thick cuticle (13 - 20 μm) and a mono-stratified epidermis (117.33 - 120.07 μm) containing calcium crystals [29] [30]. Oxalate crystal size increases as a function of maturation [29] [31] [32]. Engineered calcium oxalate crystal is suggested to confer insect resistance [15]; it affects *Bamisiatabacai* feeding choice [33]. Both oxalate crystals and oxalic acid inhibit the sucking of brown planthoppers [22]. Calcium oxalates can affect digestion and harm the mouthparts of insects [24] [34] [35]. *Opuntia* cultivars have different sizes of cuticle and epidermis [36] [37] that could also be a barrier to *Dactylopius* spp [8] [12] [38]. From the above pieces of evidence, it can be the hypothesis that calcium oxalate and other cladode features may have a role in *D. coccus* resistance of *O. ficus-indica* cultivars. Thus, this study was conducted to investigate the role of calcium oxalate, epidermis thickness, cuticle thickness, and other cladode features in *O. ficus-indica* resistant cultivars to *D. coccus*.

2. Materials and Methods

2.1. Experimental Design and Treatments

Experimental analysis was carried out at the Biotechnology Laboratory of the Botany and Zoology Department of the University Center of Biological and Agricultural Sciences of Guadalajara University in 2020. The experiment consisted of six cultivars, which are three *O. ficus-indica* (resistant 'Rojo Pelón' and susceptible 'Atlixco' and 'Chicomostoc'), two *O. cochenillifera* (resistant 'Bioplástico' and susceptible 'Nopalea') and one *O. robusta* (resistant 'Robusta'), arranged in a completely randomized design (CRD) with three replications. These cultivars were chosen based on their resistance or susceptibility to the insect *D. coccus* *[14]*. Cladodes of the study cultivars were collected from several production farms in different locations of Mexico (Guadalajara, Jalisco; Ojuelos, Jalisco; and Villanueva, Zacatecas). Matured, vigorous, and free of plagues and diseases cladodes were selected for taking samples. Cladode length and width were measured with a ruler; thickness was measured with Vernier caliper, and weighing balance was used to measure cladode weight.

2.2. Sample Preparation Examining

Samples were taken from the middle part of the cladodes, which consisted of embedding the samples in polyethylene glycol (PEG) 1450 M mass in a 1:4 proportion (PEG: deionized water) according to [39], with a modification that boiled samples instead of fresh ones. A rotatory microtome was used to obtain 15 μm sections from the samples in PEG; then, they were stained with a double treatment using safranin 0.5% (1:1 w/v) and 0.5% toluidine blue (1:1 w/v). A light microscope (with a magnification level of 10X) was used to analyze the tissues. Anatomical data; cuticle thickness, epidermis thickness, and oxalate crystals number were recorded. Measurement of the epidermis (EP), respective cuticles (CU), and oxalates number per 1 mm length were performed using the software ImageJ.

2.3. Data Analysis

The results were subject to Analysis of variance (ANOVA) and correlation with statistical software package R.4.2.0. Least significant difference (LSD) test at (0.05) was applied to compare means among treatments. Paired t-test was also done to compare resistant and susceptible groups. The association of cladode characteristics and insect establishment was done using Pearson's correlation coefficient.

3. Results and Discussion

3.1. Cladode Characteristics of Different Cultivars of *Opuntia*

The cultivars showed a very highly significant difference in cladode weight (g) (P = 0.0034), width (cm) (P = 0.0033), and thickness (cm) (P = 0.0000); and a significant difference in cladode length (cm) (P = 0.0104) (Table 1). In line with this finding, differences in cladode morphological characteristics (width, length, and thickness) of *Opuntia* spp [40]) and *O. ficus-indica* cultivars have been reported [41].

3.2. Anatomical Characteristics of Different Cultivars of *Opuntia*

Means of epidermis thickness and number of calcium oxalate showed very highly statistically different (P = 0.0000), but cuticle thickness did not show a statistically significant difference (P = 0.3660). 'Bioplástico' followed by 'Robusta' and 'Rojo Pelón' showed the highest number of calcium oxalate crystals. Similarly, 'Bioplástico' followed by 'Robusta' and 'Rojo Pelón', demonstrated higher epidermis thickness than the other cultivars (Table 2).

Table 1. Means ± SE of cladode weight (CWt) in g, length (CL), width (CW), and thickness (CT) in cm of six *Opuntia* cultivars.

Cultivar	Resistance	CWt	CL	CW	CT
Rojo Pelón	R	1260.00 ± 174.74^{c}	36.00 ± 3.78^{bc}	17.3 ± 1.20^{bc}	3.50 ± 0.14^{d}
Atlixco	S	863.00 ± 220.48^{abc}	39.00 ± 2.67^{c}	19.7 ± 3.18^{c}	2.20 ± 0.14^{b}
Chicomostoc	S	986.67 ± 114.06^{c}	38.67 ± 2.60^{c}	18.0 ± 1.16^{c}	2.33 ± 0.17^{bc}
Bioplástico	R	420.00 ± 52.99^{bc}	33.00 ± 2.64^{bc}	11.8 ± 0.44^{ab}	2.42 ± 0.08^{bc}
Nopalea	S	190.00 ± 52.92^{a}	28.67 ± 2.31^{ab}	9.7 ± 0.34^{a}	1.59 ± 0.08^{a}
Robusta	R	900.00 ± 208.16^{c}	24.67 ± 1.85^{a}	23.0 ± 30^{c}	2.67 ± 0.08^{c}

Means sharing the same letter are not significantly different at P ≤ 0.05. SE Standard error; R-resistant; and S-susceptible.

Table 2. Means ± SE results of epidermis thickness, cuticle thickness, and calcium oxalates number of six *Opuntia* cultivars.

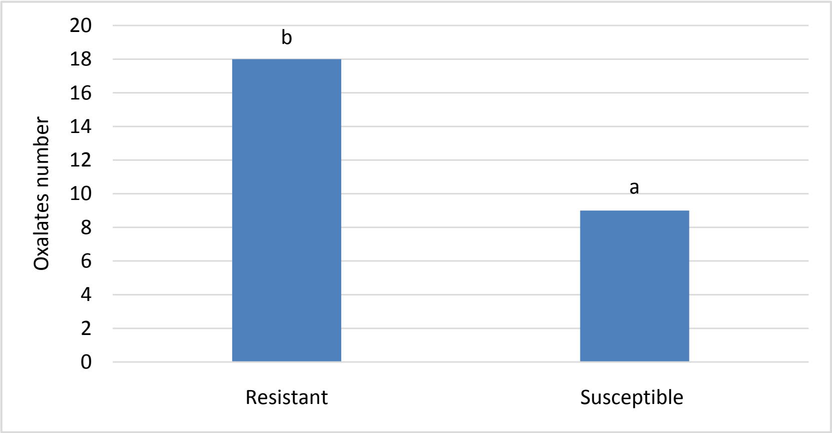
Cultivar	Oxalate number	Epidermis thickness	Cuticle thickness
Rojo Pelón	15.9 ± 0.34^{b}	0.18 ± 0.026^{abc}	0.0293
Atlixco	9.0 ± 0.23^{a}	0.13 ± 0.006^{a}	0.0223
Chicomostoc	9.3 ± 0.88^{a}	0.14 ± 0.010^{ab}	0.0470
Bioplástico	20.7 ± 2.08^{c}	0.21 ± 0.032^{c}	0.0281
Nopalea	8.9 ± 0.66^{a}	0.13 ± 0.007^{a}	0.0230
Robusta	18.9 ± 2.31^{bc}	0.19 ± 0.014^{bc}	0.0233

Means sharing the same letter are not significantly different at P ≤ 0.05. SE Standard error.

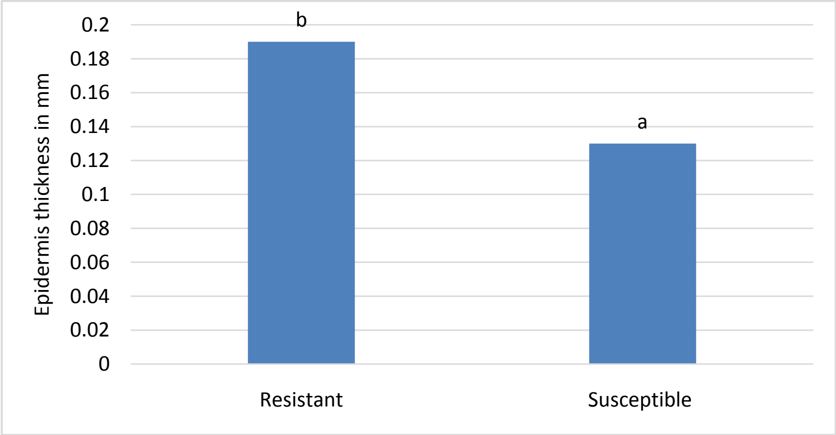
The resistant groups showed significant differences and double the number of calcium oxalate (18) than the susceptible group which scored 9 (P = 0.0000), and the average epidermis thickness of the resistant and susceptible cultivars was 0.19 mm and 0.13 mm respectively, with significant differences (P = 0.0015) (**Figure 1**).

The calcium oxalates observed are circular crystals (druse) [38]. Differences in the density of calcium oxalate crystals were reported among 15 cactus pear cultivated species of *Opuntia*, which ranged from 18 up to 57 per mm^2 [16]. The layer of calcium oxalates hampers the insertion of the stylets of *D. opuntiae* into the host plant [8]. Calcium oxalate crystals have negative effects on the growth of *Spodoptera exigua*, and the larvae also prefer to feed on *Medicago truncatula* lacking calcium oxalate [42]. They are also toxic to insects including sub-sucking [23] [24] [43] [44] [45]. This is because calcium oxalate crystals seem to serve as a feeding deterrent to insects [15] [19] [35]. It reduces growth rate and increases insect mortality; and hampers ingestion [35] [43] [46].

The difference in epidermis thickness is also supported by previous similar



(a)



(b)

Figure 1. Chart showing the average number of oxalate crystals (a) and epidermis thickness in mm (b) of the resistant and the susceptible *Opuntia* cultivars to *Dactylopius coccus*.

research. The difference in epidermis thickness is also supported by previous similar research. 2 - 3 µm width of the cuticle, 6 to 10 µm width of the epidermis, and difference among *Opuntia* spp were observed [36]. [16] mentioned that the epidermis was the main anatomical barrier to *D. opuntiae*, providing greater resistance and integrity of the cladode and varying among studied cacti. The author cleared that those cacti with thick epidermis are insect resistant. The thickness of the epidermis and cuticle can be good resistance factors of *Opuntia* spp to the cochineal (*D. opuntiae*) [38].

From **Figure 2**, it can be depicted that calcium oxalate crystals seen as spots are denser at the resistant cultivars (a) to (c) than at the susceptible cultivars (d) to (f). Pearson's correlation coefficient analysis showed that cladode thickness (0.68), calcium oxalate number (0.89), and epidermis thickness (0.75) have a significant positive correlation with *D. coccus* resistance (**Table 3**).

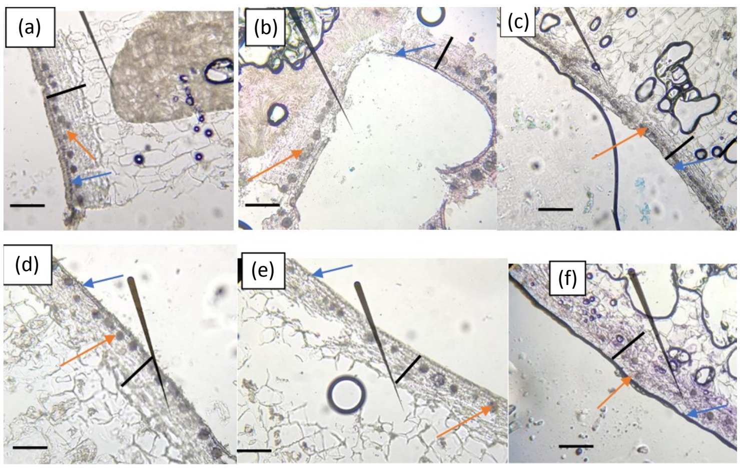


Figure 2. Microscopic view of histological cuts of cladodes showing calcium oxalates (indicated with red arrow), the epidermis (indicated with black solid line), and cuticle (indicated with blue arrow) of six *Opuntia* cultivars, (a) 'Rojo Pelón', (b) 'Robusta', (c) 'Bioplástico', (d) 'Atlixco', (e) 'Chicomostoc', and (f) 'Nopalea' detected with a microscope at a magnification level of 10X. Bars = 110 µm.

Table 3. Pearson correlation among different morpho-anatomy of cladodes of six *Opuntia* cultivars and *Dactylopius coccus resistance*.

	R	CL	CW	CT	CwT	OxN	CuT	EpT
R	1	−0.35	0.15	0.68*	0.22	0.89*	−0.13	0.75*
CL		1	0.23	0.19	0.51*	0.33	0.27	−0.08
CW			1	0.47*	0.80*	0.06	0.03	0.48
CT				1	0.76*	0.44	−0.03	0.48*
CwT					1	0.02	0.16	0.09
OxN						1	−0.07	0.77*
CuT							1	−0.08
EpT								1

*Significant at P ≤ 0.05 probability level. R-resistance; CL-Cladode length; CW-Cladode width; CT-Cladode thickness; CWt-Cladode weight; OxN-Oxalates number; CuT-Cuticle thickness, and EpT-Epidermis thickness.

In this research, the calcium oxalate crystals and epidermis thickness in cladodes of *O. ficus-indica* appeared to have a strong association with resistance against *D. coccus.* Similar previous research done by [47] on sap-sucking insects affirmed these findings. Active host-plant resistance mechanisms against aphids involve resistance factors based at, or within, the leaf epidermis or within the phloem sap, such as defense chemistry, reduced nutritional content, and lower palatability [48]. Other authors also discussed that resistant mechanisms of *Panicum virgatum* (Poaceae) against *Sipha flava* (Aphididae) could reduce amino acid content and higher oxalic acid levels. Calcium oxalate is the precipitation form of oxalic acid [49] and increased concentrations of this acid promote calcium oxalate crystals formation [50].

Due to the reduction of nutritional value [51], the presence of these crystals in plants is unfavorable for herbivores and has negative effects on sucking insects [24] [43] [52]. Calcium oxalate crystals may be also sufficient to have some detrimental effects on insect fitness by damaging their mouthparts [24]. [16] stated that a high concentration of calcium oxalate crystals prevents nymphs from inserting their stylets and establishing themselves on the cladodes. [53] concluded that in conifer stems the patterns and frequency of calcium oxalate crystals function as a constitutive defense and in combination with fiber rows, provide an effective barrier against small bark-boring insects. An increased number of calcium oxalate enhanced insect resistance in *Prunus avium* cultivars [54]. Orchids recruited the strongest defensive strategies, consisting of a thick epidermis, a larger proportion of needle-like calcium oxalate crystals, and higher content of alkaloids and quinones [55], indicating that plants can use one or different physicochemical defense mechanisms [56].

In this study 'Rojo Pelón', an *O. ficus-indica* and *D. coccus* resistant cultivar found with a greater number of calcium oxalate crystals, originated from Northern Guanajuato and Southern San Luis Potosi in the Central Highlands of Mexico [57] [58]. This cultivar is a vigorous plant with sweet, bright red, and export-quality fruits and for having spineless cladodes it is useful as forage [58]. It could be a good candidate as an alternative to those regions in the world including Tigray, Ethiopia, where resistant *Opuntia* cultivars to *D. coccus* are needed.

4. Conclusion

The number of calcium oxalate crystals (druses) and epidermis thickness was found to be higher in resistant cultivars and positively correlated with resistance as in previous works also confirming that the number of oxalates and epidermis thickness can contribute to insect resistance either by deterring or toxifying the insect. Further study could be important to assess other resistant contributing factors and the plant-insect reaction during the feeding process. Using resistant varieties is an important integrated pest management strategy for the environment and for the producers.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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