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# Detection of insecticide resistance in field populations of citrus mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae)

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*Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), commonly called citrus mealybug, is a major sporadic pest and found to cause severe yield loss in many fruit crops. Mealybugs are considered "hard to kill pests" using insecticides. In order to study the evolution of insecticide resistance in *P. citri*, we collected mealybugs from four major fruit crops and bioassay was carried out against four commonly used insecticides belonging to different groups followed by quantification of enzymes. The mortality study revealed evolution of very low to low level of resistance with resistance ratio(RR)-ranging from 6.87-14.58-fold (acephate), 7.46-16.39-fold (dichlorvos), 2.00-9.50-fold (imidacloprid) and 9.83-12.75-fold (buprofezin). Elevated levels of detoxifying enzymes were observed in all field collected populations along with the evidence of significant positive correlation between insecticides (OP, imidacloprid and buprofezin) and enzymes (esterase, MFO and GST). The high selection pressure of acephate, dichlorvos and buprofezin in the field collected population could be attributed to the evolution of low level resistance resulting in control failure. Hence, it is suggested to use imidacloprid supplemented with biocontrol strategies for the management of *P. citri.*

**Keywords:** Acephate Biological pest control, Buprofezin, Dichlorvos, Imidacloprid, IPM, Pest control

Mealybug *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) is considered as soft-bodied, small plant-sucking insect pest and is of main concern due to its presence on horticultural crops intended for export markets[1]. The family Pusedococcidae consist of about 2000 species of mealybugs belonging to 300 genera[2].

Among mealybugs, *P. citri* is a major sporadic pest of interior plants scapes and green house environments[3]. *Planococcus citri* acts as a vector for the viral disease GLRaV-3 which results in leaf roll, a serious grapevine disease spread throughout the world and cause yield loss with an average of 15-20%[4]. *Planococcus citri* was found to damage approximately 250 plant species in 90 families[5]. It was reported as a major pest of coffee arabica, robusta, mango, carambola, cotton, cocoa, banana and ginger[6].

The control of mealybugs on many crops has relied on the use of insecticides and their intensive use over the years has led to the evolution of resistance and thrashing of biodiversity. In India, 65% of insecticides are being used for the control of insect pests and 14% is being used on fruits and vegetable crops[7]. Due to the perennial nature of fruit trees, the repeated application of insecticides is in practice[8]. Chlorpyriphos, diazinon, acephate, dichlorvos, biopesticides, neonicotinoids, and insect growth regulators with new mode of action are still being used for the effective control of mealybugs.[9]

In general, resistance is mediated by behavioural changes, physiological modifications and also metabolic detoxification[10]. The most common insecticide resistance mechanism in insects is enzyme based with increased metabolic detoxification, where the degradation of xenobiotics is mediated by three major enzymes namely, cytochrome P450 (CYPs), esterases (EST) and glutathione S transferases (GST)[10]. Several agricultural and horticultural important pests have developed resistance with enhanced enzyme activity to various groups of insecticides[11].

There is no systematic study done on determination of insecticide resistance and of detoxifying enzymes in *P. citri* in India. Therefore, in this study, we collected different *P. citri* populations from four major crops in various locations to detect the

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evolution of insecticide resistance and understand the possible role played by the detoxifying enzymes.

## Materials and Methods

### Mealybugs

Different populations of *P. citri* were collected during 2014-2015 and the collection history is given in Table 1. Based on morphological keys[12] mealybugs were identified and pooled from the respective locations and were further reared on pumpkin (*Cucurbita moschata* Duchesne *ex poir*) population-wise to get uniform stage for the bioassay. The population which was initially collected from *Psidium guajava* L. from Bangalore was continuously reared for 45 generations without exposing to insecticides in the laboratory and used as a susceptible population.

### Insecticides and chemicals

Commercial grades of insecticides commonly used for control of mealybugs *viz*., organophosphates [acephate (Asataf 75% SP), dichlorvos 75% SP], neonicotinoid-imidacloprid (Confidor 17.8 SL) and insect growth regulator-buprofezin (Applaud 25 SC) were selected and used for the bioassay. The following chemicals *viz*. $\alpha$-naphthol, fast blue B salt, $\alpha$-naphthyl acetate, bovine serum albumin (BSA), reduced glutathione, 1-chloro 2,4-dinitrobenzene (CDNB), *p*-nitroanisole, nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) and *p*-nitrophenol (Sigma Chemical Co., USA) were used for bio-chemical studies.

### Bioassay

Range test was performed according to the dosage mentioned by Insecticide Resistance Action Committee (IRAC) with each insecticide for three generations prior to the final bioassay for bracketing of insecticide dilutions. Six to ten serial dilutions as gm/L of the active ingredient of the insecticides were prepared using distilled water. The bioassay was carried out according to Prabhaker *et al*.[13], where they used different methods for contact and systemic insecticides.

*Petri dish bioassay*

Susceptibility to contact insecticides that are applied on leaves was assessed using petridish technique in which young *P. guajava* leaves were cut according to the size (5-cm diameter) of the petriplate and were dipped in different dilutions of insecticides for 10 s. Treated leaves were dried in air for 30 min and ten third instar stage of *P. citri* female were released on to each leaf disc placed on 1.5% agar bed in dishes (15 cm diameter). Three replicates with 10 mealybugs in each replication were used with respective insecticide concentration. Similarly, cut leaves dipped in the distilled water were used as control and the same numbers of mealybugs were released.

*Systemic bioassay*

Baseline toxicity data of imidacloprid was determined using a systemic uptake technique as described by Prabhaker *et al*. (2012)[13]. Petioles were detached from *P. guajava* plant and infested with third instar (female) of *P. citri* and were placed in aquapiks containing serial dilutions of imidacloprid. Uptake of each concentration solution was allowed through petioles for 48 h to ensure distribution in the leaf through the stem. Control leaves infested with mealybugs were allowed for uptake of distilled water.

Entire bioassay study was conducted at a constant temperature of 25 ±2ºC; 65±5% RH with a photoperiod of 14/10 light/dark in the BOD incubator (Model no: REMI CI-12S, INDIA). The mortality was observed after 48 h exposure to insecticides and if there was any failure in movement of nymphs after gentle touch with the brush, were considered as dead.

### Biochemical analysis of enzymes

*Enzyme extracts preparation*

For EST and GST activities, groups of five third instar female nymphs were homogenised on ice in 250 µL of phosphate buffer (0.1M, pH 7.5) containing 0.1% triton×100 using a motorised pestle. The homogenate was then centrifuged at 15,000×g for 15 min at 4ºC and the supernatant was used as enzyme source. For MFO activity, groups of ten third instar nymphs were homogenised in 1 ml of 0.1 M phosphate buffer (pH 7.5) containing 10% glycerol, 0.1 mM DTT,1 mM PTU, 1 mM PMSF and 1 mM EDTA, then centrifuged at 15,000 ×g for 10 min at

Table 1—Details of *Planococcus citri* populations collected on different host plants

| Host | Location | GPS coordinates | History of insecticide used |
|---|---|---|---|
| *Vitis vinifera* | Bengaluru rural | $12.9811^0$N, $77.5746^0$E | buprofezin, imidacloprid, dichlorvos, acephate |
| *Psidium guajava* | Dharwad | $15.4589^0$N, $75.0078^0$E | phosphamidon, dichlorvos, buprofezin |
| *Manilkara zapota* | Kolar | $13.177^0$N, $78.2020^0$E | phosalone 35 EC, chlorpyriphos 20 EC |
| *Annona reticulata* | Bijapur | $16.8302^0$N, $75.1700^0$E | buprofezin, imidacloprid, malathion |
| Susceptible population | Chikkaballapur | $13.5229^0$N, $77.8367^0$N | |

4ºC and supernatant collected was used as enzyme source. The crude enzyme extract was used for total protein estimation and poly acrylamide gel electrophoresis (PAGE). Three replications were made for each of the enzyme assay.

*Esterase assay*

Total protein content of the enzyme solution was determined by Lowry's method[14]. Activity of the enzyme was determined[15] and was expressed as µmoles of α-napthol formed /min/ µg of protein. All samples were replicated three times. Native PAGE was performed using a Bio-Rad vertical slab system with 7.5% separating/resolving gel and 3% of stacking spacer gel to study esterase isozymes from the homogenates of susceptible and resistant populations of *P. citri.*

*GST assay*

Protein estimation was done by Lowry's method[14] using bovine serum albumin as a standard prepared at 1mg/ml. Specific activity of glutathione S-transferase was determined[15]. Activity was calculated with an extinction coefficient of 9.6 mM/cm for CDNB. Specific activity of enzyme was calculated and expressed as µ moles of CDNB conjugated /min/mg of protein.

*MFO assay*

A standard curve was prepared by taking absorbance of different dilutions with 20 mM stock solution for determining unknown concentration from the curve. Each dilution was made with 0.5N NaOH and the absorbance was read at 405 nm. MFO activity in *P. citri* was measured with *p*-nitroanisole as substrate[15].

**Statistical analysis**

Dosage mortality results were corrected by Abbott's formula[16] and subjected probit analysis[17] using POLO (LeOra software 1987). Significant differences were inferred by non-overlapping of 95% fiducial limit. The resistance ratio (RR) was calculated according to the Robertson and Preisler (1992) formula[18]. The classification of resistance levels was done as, RR value <10-fold as very low resistance, RR = 10-20-fold as low resistance, RR = 21-50-fold as moderate resistance, 51-100-fold as high resistance and >100 fold as significantly high resistance[19]. Data on detoxification enzyme activities was subjected to analysis of variance (One way ANOVA) using the software Statistical Package for the Social Sciences (SPSS) Version 16. The activity ratios for the three enzymes were calculated as the quotient between mean activity in the field and susceptible populations. The relationship between resistance ratio to various insecticides and enzyme activity ratios were analysed by Pearson's correlation using SPSS 16.0.

## Results and Discussion

Mealybugs are serious threat for the successful cultivation of many agricultural and horticultural crops and insecticides are commonly used for their management. Therefore, there could be a control failure which forces farmers to go for repeated sprays of insecticides and which may further result in evolution of resistance.

The concentration mortality study of *P. citri* for acephate is given in Table 2. The population collected from *P. guajava* showed high $LC_{50}$ (0.452 gm/L) compared to the susceptible population. All field collected *P. citri* populations showed very low to low level of resistance with the RR ranging from 6.87 to 14.58–fold (Fig. 1). The population collected from *P. guajava* and *Vitis vinifera* showed low level resistance with RR 14.58- and 11.80-fold, respectively. All the field collected populations were significantly higher than that of susceptible population (Non overlapping of 95% CI, $P$ <0.05).

The toxicity results of dichlorvos against field collected populations of *P. citri* are shown in Table 2.

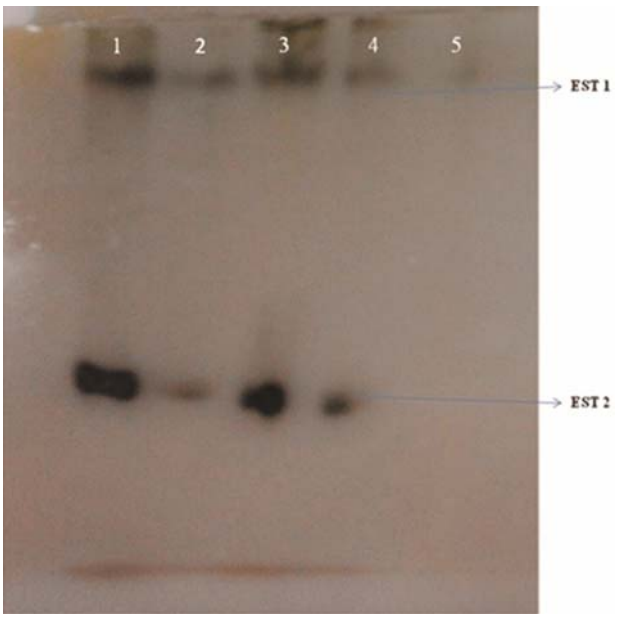


Fig. 1—Resistance ratio of field collected *Planococcus citri* population against different insecticides

Table 2—Toxicity of field and laboratory reared population of *Planococcus citri* to representative organophosphates, neonicotinoid and insect growth regulator

| Populations | $\chi^2$ | df* | Slope±SE | $LC_{50}$ (95%FL) gm/L | P** | n*** |
|---|---|---|---|---|---|---|
| | | | Acephate | | | |
| *Psidium guajava* L | 0.26 | 6 | 1.135±0.180 | 0.452 (0.235-0.748) | 0.9 | 240 |
| *Vitis vinifera* L | 2.07 | 6 | 1.105±0.159 | 0.366 (0.215-0.567) | 0.91 | 240 |
| *Manilkara zapota* L | 0.98 | 6 | 1.053±0.164 | 0.327 (0.169-0.539) | 0.91 | 240 |
| *Annona reticulata* L | 0.93 | 6 | 0.659±0.137 | 0.213 (0.069- 0.441) | 0.84 | 240 |
| Susceptible population | 2.49 | 6 | 1.012±0.203 | 0.031 (.007-0.065) | 0.99 | 240 |
| | | | Dichlorvos | | | |
| *Vitis vinifera* L | 0.75 | 6 | 0.840±0.152 | 0.528 (0.249-0.979) | 0.82 | 240 |
| *Psidium guajava* L | 0.32 | 6 | 0.852±0.152 | 0.459 (0.214-0.842) | 0.84 | 240 |
| *Manilkara zapota* L | 2.2 | 6 | 1.294±0.186 | 0.424 (0.247-0.654) | 0.93 | 240 |
| *Annona reticulata* L | 0.84 | 6 | 0.771±0.140 | 0.209 (0.078-0.407) | 0.87 | 240 |
| Susceptible population | 2.5 | 6 | 0.985±0.202 | 0.028 (0.006-0.060) | 0.99 | 240 |
| | | | Imidacloprid | | | |
| *Vitis vinifera* L | 1.34 | 4 | 0.770±0.203 | 0.038 (0.015-0.085) | 0.73 | 180 |
| *Psidium guajava* L | 1.5 | 4 | 1.004±0.212 | 0.015 (0.006-0.026) | 0.88 | 180 |
| *Manilkara zapota* L | 1.74 | 4 | 1.133±0.232 | 0.013 (0.005-0.023) | 0.92 | 180 |
| *Annona reticulata* L | 0.47 | 4 | 1.121±0.234 | 0.008 (0.003-0.014) | 0.94 | 180 |
| Susceptible population | 2.54 | 4 | 1.003±0.239 | 0.004 (0.001-0.008) | 0.96 | 180 |
| | | | Buprofezin | | | |
| *Vitis vinifera* L | 1.11 | 8 | 0.857±0.161 | 0.625 (0.261-1.265) | 0.81 | 300 |
| *Manilkara zapota* L | 0.49 | 8 | 0.945±0.177 | 0.559 (0.239-1.057) | 0.84 | 300 |
| *Annona reticulata* L | 1.74 | 8 | 0.709±0.132 | 0.532 (0.197-1.247) | 0.78 | 300 |
| *Psidium guajava* L | 1.34 | 8 | 0.756±0.138 | 0.482 (0.184-1.062) | 0.81 | 300 |
| Susceptible population | 1.15 | 8 | 0.771±0.113 | 0.049 (0.019-0.093) | 0.94 | 300 |

[*Degrees of freedom; **Probability; ***Number of female nymphs used in the bioassay including control]

*P. citri* population collected from *V. vinifera* showed high $LC_{50}$ (0.528 g/L) followed by *P. guajava* (0.459 g/L). All field collected populations of *P. citri* showed very low to low level of resistance. High resistance among the tested population was shown by *P. citri* collected on *V. vinifera* having RR of 18.85-fold (Fig. 1). There was no significant difference among the field collected population (Overlapping of 95% CI, $P$ <0.05).

The mortality response shown by the *P. citri* to different concentrations of imidacloprid is shown in Table 2. Population collected from *V. vinifera* showed 9.5–fold of RR (Fig. 1). With respect to imidacloprid, no significant difference was found between field and susceptible populations (Overlapping of 95% CI, $P$ <0.05). The dosage mortality test of buprofezin against *P. citri* is given in Table 2. In general, the field collected populations showed higher $LC_{50}$ values ranging from 0.049-0.625 g/L. The highest RR found in the population collected from *V. vinifera* (12.75-fold), showing evidence for the evolution of low level of resistance. There was significant difference between populations collected from *V. vinifera*, *Manilkara zapota* and populations collected from *Annona reticulata*, *P. guajava* (Non overlapping of 95% CI, $P$ <0.05).

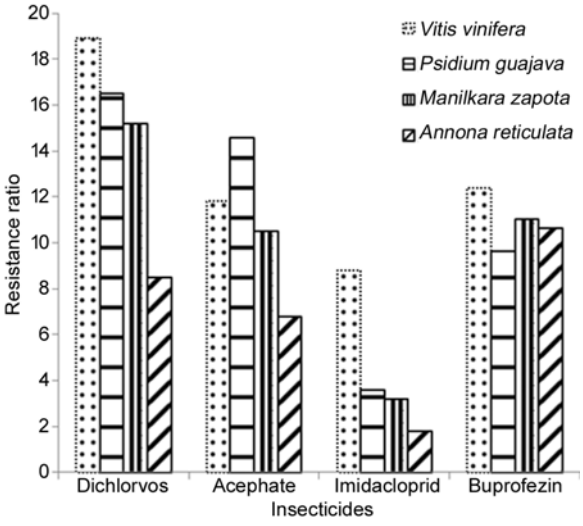


Fig. 2— (A) Esterase; (B) Glutathione S transferase; and (C) MFO activity in different field collected populations of *Planococcus citri*. [A, B & C expressed in µmoles of α-naphthol, µ moles and µ moles of p-nitro phenol/min/ mg of protein, respectively]

The esterase activity for field collected and susceptible population ranged between 0.056 to 0.219 µmoles of α-naphthol/min/mg of protein. The elevated level of esterase activity was observed in the population collected from *V. vinifera* followed by *P. guajava* (Fig. 2A). One way ANOVA analysis

Table 3—Correlation coefficients (r) between toxicity of insecticides and enzyme activities in *Planococcus citri*

| Insecticides | GST | EST | MFO |
|---|---|---|---|
| Acephate | 0.962*(0.038) | 0.991** (0.009) | |
| Dichlorvos | 0.962*(0.038) | 0.991** (0.009), 0.986*(0.014) | 0.960* (0.040) |
| Imidacloprid | 0.962* (0.038) | 0.962* (0.038) | |
| Buprofezin | 0.962* (0.038) | 0.962* (0.038), 0.986* (0.014) | |

[The figures in parentheses indicate the probability rejecting null hypothesis that r = 0,*represents significant correlation at $P$ <0.05 and ** represents significant correlation at $P$ <0.01 level]

showed significantly higher activity of esterase in all field collected populations compared to the susceptible population with the activity ratio ranging between 2.4 to 3.9-fold. Pearson's correlation analysis (Table 3) showed a significant positive correlation between esterase, and all tested insecticide in the study (acephate, dichlorvos, imidacloprid, buprofezin) ($R^2$ = 0.991, $P$ <0.01). The activity of esterase visualised on native PAGE showed two prominent bands and were named as Est-1 and Est-2 based on their distance moved in the gel. Two distinct bands among the field collected populations were observed, whereas in susceptible population (Fig. 3), only a single faint band was observed. Qualitatively there was no much difference among the banding pattern of esterase among four field collected populations.

The GST activity ranged between 0.115 to 0.529 µmoles/min/mg of protein. The highest activity recorded in the population collected from *V. vinifera* (0.529 µmoles/min/mg of protein). The activity ratios were in the range of 2.3 to 4.6-fold (Fig. 2B). One way ANOVA analysis showed significantly ($P \geq 0.01$) higher activities of GST among the field population compare to susceptible population. Pearson's correlation analysis (Table 3) showed significant positive correlation between GST and OP compounds ($R^2 = 0.962$ $P \leq 0.05$).

There was an increase in the activity of MFO in the field collected populations as compared to susceptible population. Elevated activity level was observed in population collected from *V. vinifera* (4.583 µmoles of *p*-nitro phenol/min/mg of protein) with 3.9 fold increase in the activity ratio (Fig. 2C). One way ANOVA analysis showed a significant difference in the activity in all field collected populations ($P \geq 0.01$). Pearson's correlation analysis (Table 3) showed a significant positive correlation with dichlorvos ($R^2 = 0.960 P \leq 0.05$).

In the present study, out of four populations of *P. citri*, the population collected on *V. vinifera* showed

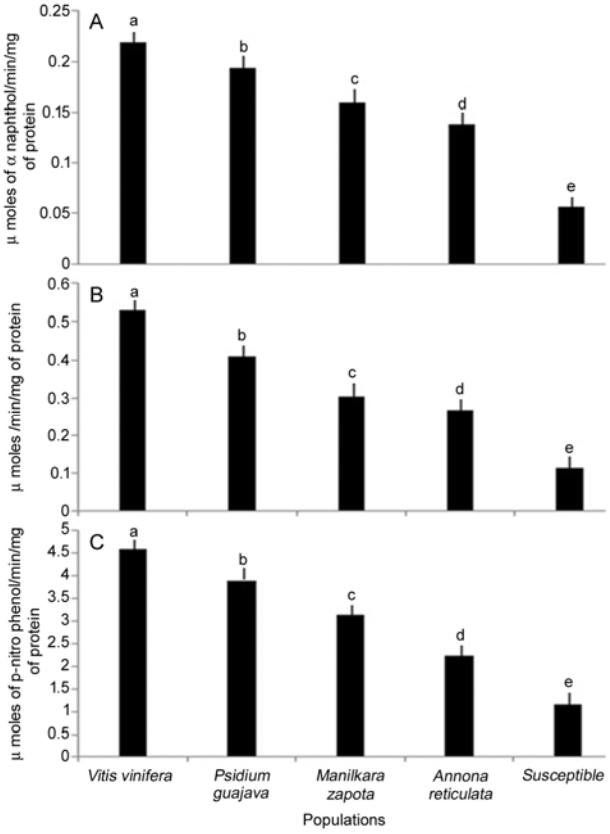


Fig. 3—Native PAGE for different populations of *Planococcus citri*. [Lanes: 1, *V. vinifera*; 2, *A. reticulata*; 3, *P. guajava*; 4, *M. zapota*; and 5, Susceptible. EST, Esterase]

low level of resistance to all the group of insecticides (dichlorvos, acephate, imidacloprid and buprofezin). During the survey to farmer's field, it was found that more of organophosphates (dichlorvos, acephate, phosphamidon, chlorpyriphos and malathion) were used to control mealybugs along with imidacloprid and buprofezin. If more than 10-fold of resistance ratio (RR) was observed, then the insects are considered as resistance[19]. Present results showed low levels of resistance against dichlorvos for the population collected from *V. vinifera* followed by *P. guajava*, *M. zapota* and *A. reticulata*. The incidence of mealybug has become one of the major concerns on *A. reticulata* in Maharashtra and Karnataka[20] and *P. citri* is considered as one of the major pest on *A. reticulata* along with *Ferrisia virgata* (Cockerell) and *Maconellicoccus hirsutus* (Green). Furthermore, population from *P. guajava* showed low level of resistance to acephate followed by *V. vinifera* and *M. zapota*.

The first report of resistance in mealybug dates back to 1964, against parathion in USA and South

Africa, and it was suggested that multivoltinous nature of mealybug and repeated practice of inefficient control tactics hasten the evolution of resistance. Present result signifies the gradual evolution of resistance to OP. Though the resistance is at very low levels, there is a possibility that repeated usage of broad spectrum of insecticides might have resulted in the evolution of resistance to other OP. In few parts of Taiwan, OP (methidathion, formothion and dimethoate) at various rates gave effective control of *P. citri* with 2-3 times spray at 7-10 days interval[21]. Since then, not much work has been done on the development of resistance to different group of insecticides. Effective control of mealybugs on citrus, guava and grapes[22] was achieved using acephate, profenophos, thiamethoxam and chlorpyriphos. The extensive and regular use of insecticides on various fruit crops in India could have escalated the risk for the potential evolution of resistance in *P. citri.*

Control of mealybugs on custard apple was not achieved using dichlorvos and imidacloprid when compared to buprofezin and chlorpyriphos[20,23]. In the present study, imidacloprid gave a better control of *P. citri*, where the $LC_{50}$ as well as the RR were less when compared to the other insecticides used. Similar results were reported in *Planococcus ficus* Signoret[13], where imidacloprid gave a better control. With respect to *Phenacoccus solenopsis* Tinsley*,* 315-fold[24] and 105 -fold[25] of resistance was reported against acetamiprid which is a neonicotinoid. A recent study with the usage of imidacloprid with 4x greater than the label claim to control citrus mealybug feeding on coleus (*Solenostemon scutellarioides* [L.] Codd) plant showed 35% of mortality, where as for other systemic insecticide azadirachtin, cyantraniliprole, dinotefuran, spirotetramat, and thiamethoxam which were used preventatively resulted in mortality ≤22%[26].

Mealybugs have potential to evolve resistance to buprofezin and hence was recommended around the world to be used twice a year[1]. But according to the survey during the study, farmers when they do not get good control of mealybugs, they will go for repeated sprays which in turn results in evolution of resistance. It was stated that *P. citri* was significantly reduced by buprofezin[27] and it was also reported that buprofezin could not give good control of mealybugs in vineyards even after the first and second application following the treatment with prothiophos either on the leaves or on bunches[28]. With respect to *P. solenopsis*, resistance was reported with 34- and 28.9- fold to cyromazine and pyriproxyfen, respectively[25]. The present results showed evidence for the evolution of low level of resistance in *P. citri*. Furthermore, in all the selected fruit crops *P. citri* infestation occurs along with one or the other pest species which may provoke the use of many groups of insecticides and consecutively make way for the evolution of insecticide resistance. Few works stated that higher rate of application of buprofezin could result in effective control of mealybugs as compared to imidacloprid and cypermethrin[29,30]. Effective control of *P. citri* was achieved by using parasitoids *viz. Leptomastix doctylopii* Howard and *Coccidoxenoide speregrinus* Timberlake on sapota. However, the parasitoids are not active throughout the year and hence regulate only during specified period, on contrary farmers may choose for the insecticide usage[31].

Further Pearson's correlation analysis between RR and enzyme activity gave a clue for the possible role of enzymes playing a role in detoxification mechanism. Over production of non insecticides have been reported in other insects such as *Helicoverpa armigera* (Hübner) where elevated levels of esterase activity was observed in the organophosphate resistant population and also the resistant population showed more number of isoenzyme patterns in native PAGE as compared to the susceptible[32]. Further Pearson's correlation also showed a significant positive correlation between esterase and OP, imidacloprid. This signifies that there is a possibility for the esterase playing major role in conferring resistance to insecticides reported in *Myzus persicae* and *Aphis gossypii*.[11]

The association between elevated levels of GST activity and insecticide resistance to major groups of insecticides was reported in key insect pests such as *A. gossypii*[33] and *M. persicae*[34]. There was a gradual increase in the activity of GST in the present study, showing the possible role of GST being involved in conferring resistance supported with significant correlation with OP and imidacloprid. In insects, cytochrome P450 plays a main role in detoxification of insecticides and in such insects there will be over expression of P450 which in turn results in development of resistance[35]. The present experimental results showed elevated levels of MFO from the field collected populations compared with susceptible population, which is further supported by the Pearsons correlation between organophosphates and

MFO. Earlier reports on *M. persicae* and *Nilaparvata lugens* have shown strong evidence for the MFO playing a major role in resistance mechanism at genetic level[34,35]. Thus the elevated levels of MFO activity may give clue for the probable role of MFO conferring resistance in *P. citri*.

## Conclusion

The present study provides the baseline study for evolution of resistance in *Planococcus citri* to organophosphates and insect growth regulator with evidence of positive correlation between detoxifying enzymes and insecticides. Since the resistance level in mealybugs ranges from very low to low level, adequate measures should be taken to contain evolution of insecticide resistance. The farmers need to be sensitized to evolution of insecticide resistance in mealybugs to different groups of insecticides and also given awareness on possible management strategies like rotation of insecticides with different mode of action as per the label claims, use of botanical pesticides. Possibly, this is the first report on insecticide resistance in *Planococcus citri* in India.

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## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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