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# RESISTANCE AND ENZYME ASSESMENT OF THE PINK BOLLWORM, *PECTINOPHORA GOSSYPIELLA* (SAUNDERS) TO SPINOSAD

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## ABSTRACT

Resistance of the pink bollworm, *Pectinophora gossypiella* (Saunders) to spinosad was examined under laboratory conditions during 20 successive generations. After 20 generations of selection pressure by spinosad the resistance ratio was increased to 54.1 –fold. The resistance ratios fluctuated during the selection period. The highest resistance ratio was occurred in F16, it was 62.5 –fold. No cross resistance was observed between spinosad resistant colony and buprofezin, pyriproxyfen, lambda-cyhalothrin, thiamethoxam, chlorpyrifos and carbaryl. The resistance ratio decreased when piperonyl butoxide was mixed with spinosad. The activities of acetylcholine esterase and total protein were increased in spinosad resistant colony compared with the susceptible colony. These results confirmed that enzyme detoxification mechanism is considered one of the main mechanism of resistance to spinosad and the use of pesticides rotation play an important role in pesticide resistance management. These findings also, indicate that the ability of this new biopesticide (spinosad) to build up of resistance in the pink bollworm after the second generation of selection pressure. So, these results suggested that this biopesticide can be used twice only during the same season without any pest resistance.

**Key words**: *Pectinophora gossypiella*, spinosad, enzyme detoxification, cross resistance, resistance ratio.

## INTRODUCTION

Development of resistance to pesticides is generally considered to be one of the most serious obstacles to effective pest control today. The pink bollworm, *Pectinophora gossypiella* (Saunders) is the most serious pest on cotton in Egypt. It causes an enormous damage and loss of cotton yield when it neglected (El-Aswad and Aly, 2007). The larvae of the pink bollworm attack plants at the beginning of the fruiting stage causing huge losses to the cotton green bolls, fibers and seeds and accordingly great reduction in the cotton yield. This pest acquired resistance against most of conventional pesticides (Khurana and Verma, 1990). The intensive use of pesticides in agriculture and public health leads to adverse effects such as development of pesticide resistance, frequent pest out breaks and emergence of new pests. Ministry of Agriculture in Egypt is hopping to find a product safe and low hazard in the environment; with satisfactory killing power especially for the pink bollworm. So, Spinosad was chosen because it is classified by EPA as a reduced risk product and awarded the green chemical challenge award from the white house in USA, 1999 (Temerak, 2003). Replacement of synthetic insecticides by bio-rational insecticide is an universally acceptable and practicable approach worldwide. Some insecticides with novel modes of action have been introduced for controlling the pest effectively including spinosad.

Spinosad is the first member of Dow AgroScience's naturalyte class of insecticides (Thompson *et al.,* 1996). Spinosad is comprised primarily of two macrocyclic lactones; spinosyn A and D, secondary metabolites produced by the actinomycete, *Saccharopolyspora spinosa*, under natural fermentation conditions. The mode of action of spinosad is twofold; the primary target site is the nicotinic acetylcholine receptor, but the GABA receptor is also affected to some degree (Salgado, 1997).

Evolution of pesticide resistance has been identified worldwide as the most serious threat to the development of sustainable integrated pest management practices (Labbe *et al*., 2005). Conventional insecticides have been provided a long term solution to the pink bollworm problem (Henneberry, 1986). Moreover, as a result of continued massive use of certain synthetic insecticides against the cotton pest, tolerant and resistant strains have been developed (Schmutter, 1985). At present, several insects have exhibited ascending resistance to spinosad in field populations (Zhao *et al.*, 2006), and also cross resistance between spinosad and other pesticides was documented in *Spodoptera litura*, *Plutella xylostella* and *Lucilia cuprina* (Levot and Sales, 2008; Sayyed *et al*., 2008).

The aim of this work to make a good strategy for resistance management which lead to prolong the useful life of spinosad against the pink bollworm, *P. gossypiella* larvae in Egypt and knowledge the mechanism of resistance.

## MATERIALS AND METHODS

**Tested insect:** Laboratory colony of the pink bollworm, *Pectinophora gossypiella* was obtained from Bollworm Research Division, Plant Protection Research Institute, Agriculture Research Centre. This colony reared for more than ten years without any exposure to pesticides.

**Rearing of insect:** The adults of *P. gossypiella* were put in glass chimney cages and convenient by a muslin cloth as a suitable site for egg deposition. The moths were fed on sucrose solution (10%). The muslin was replaced every 3 days and the deposited eggs on the muslin were transferred to a convenient glass jars until hatching .The obtained newly hatched larvae were taken to be used in selection study. The rearing conditions of this colony were controlled at 27± 1ºC and 70 – 75 % RH with complete dark all day time. The larval instars were put in a glass tube (2 X 7 cm) and fed on a semi artificial diet as mentioned by Rashad and Ammar (1985).

**Test chemical:** Spinosad as Tracer 240 SC, contains about 85% spinosyn A and 15% spinosyn D with other spinosyns as minor impurities, was used in selection pressure study. Other pesticides (chlorpyrifos, lambda-cyhalothrin, pyriproxyfen, thiamethoxam, admiral, actara, buprofezin, and carbaryl) were used in cross resistance study (Table 1).

**Selection pressure of spinosad against the newly hatched larvae of the pink bollworm:** Newly hatched larvae were fed on the previously mentioned fresh diet containing different concentrations of spinosad. Using distilled water, the stock solutions of the tested compound was prepared to be used in each generation depending on the mortality percentages obtained during the preceding generation of pressure. In the beginning of pressure (i.e., with the parents) a wide rang of concentrations were used to detect the median lethal concentration ($LC_{50}$), also during the onset of detecting the susceptibility of each colony as well as during assessing the fold of resistance in all generations during selection. So, selection was started using low concentrations then gradually increased to avoid elimination of minor resistance gene if any. The spinosad concentrations started by 0.99 ppm with the parents (first generation) and end by 100 ppm in the twentieth generation (F20). With each generation of selection pressure, at least four concentrations were used to avoid losing the colony with using higher concentrations during the next generation. Each concentration was added to 50 gr. fresh prepared diet. This amount of treated diet was divided into three parts (Ca.16 g). Each one was poured into a convenient Petri dish (12 cm in diameter). Fourty healthy newly hatched larvae, starved for 6 hrs approximately, were gently transferred to each Petri dish using a soft brush. With this way three replicates were used for each concentration. Similar numbers of larvae were transferred to untreated diet as a control treatment. The dishes were maintained in an incubator at temperature of 27 ± 1 °C and 75 ± 5 R.H. with a complete dark all daytime. To simulate nature, after Ca. one hour, from exposing the first instar larvae to the treated and untreated diet, the larvae were transferred individually into clean and sterile glass tubes (2x7 cm) each containing a small piece of untreated diet, each tube contained one alive larva. After 5,11and 17 days, all tubes were inspected for estimating the mortality percentages. As well as resistance ratios were determined after each generation. The resistance ratios were obtained by dividing the $LC_{50}$ value of the resistance colony on the $LC_{50}$ value of the susceptible colony (i.e., parents). $LC_{50}$, slope values and their confidence limits were calculated every generation according to Finney (1971).

**Cross resistance of spinosad resistance colony and some recommended pesticides:** To detect the cross resistance between spinosad resistant colony and some recommended insecticides, many insecticides from different pesticides groups were chosen. After the end of selection (after F20) the newly hatched larvae of the spinosad resistant colony were tested against the tested pesticides. Three concentrations from all tested pesticides were used (the field concentrations and the lower two concentrations) (Table 1). These concentrations were tested also against the laboratory colony (so called susceptible colony). The rate of cross resistance was calculated by dividing of the $LC_{50}$ in resistant colony which treated by the tested pesticide on the $LC_{50}$ in susceptible colony.

**Synergistic action of the piperonyl butoxide (PBO) on the spinosad resistant colony:** The piperonyl butoxide PBO concentration was always 100 ppm. This concentration was determined by initial experiments to obtain the concentration of PBO which not causing any mortality in the susceptible colony (Zamojska *et al.* 2011). Other two lower concentrations were used (50 and 25 ppm). The last concentration of spinosad which used in the 20$^{th}$ generation (54 ppm and lower concentrations; 26 and 13 ppm) was added to the same concentration of PBO. On the basis of the results of these experiments, $LC_{50}$ and slope values were calculated. The synergism coefficient (SC) was calculated according to Zamojska *et al.* (2011) as follows:

$$SC = \frac{\text{LC50 of active substance alone}}{\text{LC50 of active substance with a synergist}}$$

The following results were accepted to estimate the synergism action between spinosad and PBO synergist:

SC < 1 – antagonism; SC = 1 – the lack of synergism and the lack of antagonism; SC > 1 – synergism.

**Enzymes assessment:** The activity of cholinesterase was

determined in both resistant and susceptible colony of the pink bollworm. The resistant larvae were homogenized in distilled water 1g insect body/5ml water using a mortar for 3min. Homogenates were centrifuged at 3000 r.p.m for 15 min under cooling centrifuge and the supernatant was used (as enzyme solution ) directly or stored at –20°C until use for biochemical determinations. A control experiment was set up using the supernatant of untreated larvae**.** Substrate specificity was similarly determined by assay against three thioesters (Weber, 1966). A 10 ul aliquot of supernatant was added to 1.5 ml of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in 52 mM phosphate buffer, pH 7.2 . After mixing and incubation, 50 ul of a 156 mM solution of a thioester acetyl thiocholine iodide was added. Enzyme activity was recorded as the increase in optical density due to conversion of DTNB to 5-thio-2-nitrobenzoic acid (Ellman *et al.,* 1961). The reaction was monitored spectrophotometrically at 405 nm, and a correction for spontaneous hydrolysis of the substrate was incorporated. The ratio of enzyme activity change was calculated by dividing the mean activity of enzyme in resistant colony on susceptible colony.

**Total protein determination:** This was determined by the biuret method (Gornall *et al*. 1949), which consists of reacting 0.025 ml of sample and 1 ml of biuret reagent in a test tube, agitated and left to stand for 30 min and measured the absorbance of colored complex formed at 550 nm and samples were prepared well for denaturing electrophoresis. The absorbance obtained was interpolated in a standard of albumin. The total protein was calculated by Protein concentration (g\dl) =

$$\frac{\text{A sample x 5}}{\text{A standard}}$$

**Statistical analysis:** Data were analyzed by analysis of variance (one ways classification ANOVA) followed by a least significant difference, L.S.D at 5% (Costat Statistical Software, 1990).

## RESULTS AND DISCUSSION

The newly hatched larvae of pink bollworm were selected against spinosad during 20 successive generations.

**Development of resistance to spinosad in newly hatched larvae of the pink bollworm, *P. gossypiella*:** Building up of resistant to spinosad was slowly in the beginning of selection (Table 2). The $LC_{50}$ was 2.54 ppm in the parents (F0). In the first generation (F1) the $LC_{50}$ was decreased to 1.63 ppm. After the fist generation, the resistance ratio increased in F2, F3, F4, F5 and F6. It was 3.92, 9.14, 19.44, 23.24 and 36.29 –fold, respectively, compared to the F0 (parents) and decreased to 14.76 –fold in F7. As a result of intensive selection pressure the resistance ratio was quickly decreased to 5.25 and 9.4 –fold in F10 and F15, respectively. Exponential increase of resistance was found in F17, it was 76.1 –fold. The $LC_{50}$ was ranged between 0.44 to 75.3 ppm during the selective period. The resistance ratios were fluctuated from generation to other. The slope values also, fluctuated and ranged between 2.36 to 3.30 during the selective period.

As a result of the intensive selection by spinosad during 20 successive generations, the larvae of the pink bollworm acquired resistance to this new pesticide. The intensive selection was eliminated the susceptible individual and the resistant one was survived. Pesticides resistance is an inevitable phenomenon so; this phenomenon also existed in other insect pests. Wang *et al*. (2009) found that after 15 generations of selection in the laboratory, the *Helicoverpa armigera* strain developed more than 20-fold resistance to spinosad. Moulton *et al.* 2000 estimated the $LC_{50}$ of second- and third-instar larvae of beet armyworm, *Spodoptera exigua* field populations. The $LC_{50}$ ranged from 0.279 to 6.14 and 0.589 to 14.0 mg spinosad/ liter, respectively. Shi *et al*. 2011 reported that spinosad, a relatively new, effective and safe pesticide, has been widely used in pest control over the last 10 years. However, different levels of resistance to this insecticide have developed in some insects worldwide. The author found that after continuous selection for 27 generations, a strain (SpRR) of the housefly developed 247-fold resistance to spinosad compared with the laboratory susceptible strain (CSS). Bielza *et al.* 2007 found that the western flower thrips, *Frankliniella occidentalis* (Pergande) which selected in the laboratory to spinosad showed a very high resistance to spinosad (356.547-fold based on $LC_{50}$ values) compared with the laboratory susceptible strain. Young *et al.* (2003) selected a laboratory strain of the tobacco budworm, *Heliothis virescens*, with topical application of technical grade of spinosad. The results showed that the $LD_{50}$ value of the selected strain was 1068 –fold greater than that of the parental generation by the 14th generation.

**Cross resistance of spinosad and some recommended pesticides in different groups of pesticides:** Cross-resistance is a very serious phenomenon in the management of pest basis of resistance mechanisms among different class of chemicals. Data in Table 3 showed that there was no cross resistance between spinosad and the tested pesticides including, buprofezin, pyriproxyfen, carbaryl, thiamethoxam, chlorpyrifos and lambda-cyhalothrin. The resistance ratios were 1.10, 1.12, 0.72, 0.70, 0.24 and 1.22 –fold, respectively. This means that the pesticide rotation is a main approach to overcome on spinosad resistance. Data in Table 4 showed that there is no cross-resistance to other insecticides including those working at the nicotinic acetylcholine receptor (thiamethoxam).

The results confirmed that no cross resistance between spinosad and all pesticides used, even thiamethoxam which belong the same mode of action. This may be due to spinosad has more than mode of action in the pink bollworm larvae. The same result was showed by Shono and Scott (2003). The author found that no cross resistance between spinosad and nicotine in the housefly, *Musca domestica*. The resistance ratio reduced from 150 to 1.2 –fold. It suggests that cross-resistance may not be a limiting factor for making use of spinosad against insect pests. Wang *et al.* 2009 found that there was no cross resistance between spinosad and chlorpyrifos. The resistance ratio reduced from 24.1 to 1.46 –fold. The same authors found that no cross resistance between spinosad and fenvalerate (pyrethroids); and methomyl (carbamates). The resistance ratios reduced from 29.6 to 1.1 and 1.0 –fold, respectively.

These results suggested that using of pesticides from different pesticide classes (carbamates, organophosphours, neonicotinoid, etc.) during the season may be reduces the rate of building up of pest resistance.

**Role of PBO in spinosad efficiency on the pink bollworm larvae:** Table 4 showed that PBO has a good effect in increasing of spinosad toxicity. The $LC_{50}$ when spinosad used only against the spinosad resistant colony was 73.7 ppm, while when spinosad mixed with PBO the $LC_{50}$ decreased to 19.2 ppm. According to this result the synergism coefficient was 3.8 –fold. The slope value also, affected. It was 3.1 and 2.1 in spinosad only and spinosad combined with PBO, respectively.

The piperonyl butoxide has a great effect in decreasing of the spinosad resistance ratio. This indicates that using of spinosad combined with PBO may increase the efficacy of spinosad in control of *P. gossypiella*. This conclusion is confirmed that cytochrome P450 monooxygenase play a major role in the resistance of *P. gossypiella* against spinosad. Increasing of slope value means that the resistant colony was more homogenous when it treated with spinosad combined with PBO. The same result was found by Wang *et al*. 2009. The author found that spinosad resistance could be partially suppressed by piperonyl butoxide (PBO). Liu and Yue (2000) found that PBO increased the spinosad toxicity to housefly of both permethrin resistance and susceptible strains. Wang *et al.* 2006 reported that the synergism bioassays indicated that the spinosad resistance in *S. exigua* was suppressed by the PBO and the synergistic ratio was 9.8. It is implied that the monooxygenase might play important roles in the resistance of the beet armyworm against spinosad.

In contrast, Zhang *et al.* (2008) found that the spinosad $LD_{50}$ values for spinosad resistant strains of *Frankliniella occidentalis* were unchanged by pretreatment with PBO. Zhao *et al.* 2002 stated that the synergist PBO did not show any synergism for spinosad in the resistant colony of *Plutella xylostella*. These results mean that resistant mechanism against spinosad is probably related with the species of pests. The profile of cross-resistance development for spinosad in the pink bollworm also could be used to help evaluate the possible metabolic.

**Enzyme assessment:**

**Effect of spinosad resistance on activity of acetylcholine esterase (AChE):** As shown in Table 5 activity of AChE in resistant colony was increased two fold approximately, compared to laboratory colony (so-called susceptible colony). It was 91.23 and 45.35 µ mole/ ml/ g tissue in resistant and susceptible colony, respectively. This means that the activity of acetyl choline esterase in resistant colony was increase to 2 times compare with susceptible one. The statistical analysis shows that there is a significant difference between the activities of acetyl choline esterase in resistant colony and susceptible one. The LSD value is 25.2.

This result confirmed that acetyl choline esterase plays an important role in spinosad resistance and also most of monooxygenase enzymes. Wang *et al.* (2006) suggested that there was relationship between change of activity of some enzymes and resistance to spinosad in the beet armyworm. Abd-Elhady and Abd El-Aal (2011) found that higher level of Acetylcholine esterase activity by 4.1- fold in the resistant colony of the pink bollworm compared to the susceptible colony.

**Effect of spinosad resistance on total protein:** The total protein content in both resistant and susceptible colony was determined (Table 5). The results show that the total protein was elevated in resistant colony compared the susceptible one. It was 17.79 ±2.95 and 4.14±0.33 g /dl/ g tissue, respectively. This means that the total protein was increase to 4.5 times approximately in resistant colony compared with susceptible one. The statistical analysis shows that there is a significant difference between the total protein in resistant and susceptible colonies. The LSD is 4.7.

The total protein content also increased. This result may be due to gene amplification. This means that resistance to spinosad increases the total protein content. The same result was found by Farghaly (2010). The author mentioned that the total protein in field colony of whitefly, *Bemisia tabaci* (which exposed to different groups of pesticides) was more than the laboratory colony.

These results confirmed that pesticide resistance is an inevitable phenomenon. The rate of resistance in pink bollworm increased gradually in the beginning of selection and sharply increased after the F4. The results recommended that spinosad can be used safely against

the pink bollworm twice during the same season without any building up of resistance. No cross resistance was occurred between pink bollworm spinosad resistance colony and some insecticides from different groups of pesticides. The rate of resistance to spinosad was reduced when piperonyl butoxide was added to spinosad. The rate of acetylcholine esterase and total protein was increased in resistant colony compared with the susceptible colony. Pesticides rotation is the very important method in pesticides resistance management. Enzyme detoxification is still play an important role in new pesticides.

**Table 1: Pesticides concentrations used in cross resistance of spinosad resistance colony**

| Pesticides | Field rate | Concentrations | | |
|---|---|---|---|---|
| | | C1 | C2 | C3 |
| Chlorpyrifos | 1 liter/feddan | 1200 | 600 | 300 |
| L-cyhalothrin | 330ml/feddan | 20 | 10 | 5 |
| Pyriproxyfen | 250 ml/feddan | 62.5 | 31.25 | 15.62 |
| Carbaryl | 1 Kg/ feddan | 2125 | 1062.5 | 531.25 |
| Buprofezin | 200 ml/ feddan | 125 | 62.5 | 31.25 |
| Thiamethoxam | 50 g/ feddan | 31.25 | 15.62 | 7.81 |

C1: the first concentration C2: the second concentration C3: the third concentration

**Table 2: Development of resistance to spinosad in the pink bollworm, *P. gossypiella* during 20 successive generations**

| Filial | Slope ± SE | $LC_{50}$ and fiducial limit 95% | Resistance ratios |
|---|---|---|---|
| $F_0$ | 2.4 ± 0.33 | 0.99 (0.84 – 1.14) | 1.0 |
| $F_1$ | 2.8 ± 0.35 | 0.44 (0.38 – 0.50) | 0.44 |
| $F_2$ | 2.69 ± 0.34 | 3.88 (3.34 – 4.44) | 3.92 |
| $F_3$ | 2.69 ± 0.34 | 9.05 (7.72 – 10.35) | 9.14 |
| $F_4$ | 2.91 ± 0.35 | 19.25 ( 16.72 – 21.79) | 19.44 |
| $F_5$ | 3.11 ± 0.35 | 23.01 (20.39 – 25.88) | 23.24 |
| $F_6$ | 3.05 ± 0.36 | 35.93 (31.06 – 40.61) | 36.29 |
| $F_7$ | 3.26 ± 0.4 | 14.61 (12.36 – 16.60) | 14.76 |
| $F_8$ | 3.05± 0.4 | 48.37 (43.05 – 54.57) | 48.86 |
| $F_9$ | 3.33±0.37 | 59.18 (59.18 – 75.28) | 59.78 |
| $F_{10}$ | 2.53± 0.33 | 5.46 (4.69 – 6.27) | 5.52 |
| $F_{11}$ | 2.36± 0.33 | 36.65 (30.31 – 42.63) | 37.02 |
| $F_{12}$ | 2.8 ±0.3 | 11.4 (9.9 – 12.9) | 11.5 |
| $F_{13}$ | 3.3 ± 0.4 | 58.2 (52.1 – 65.6) | 58.8 |
| $F_{14}$ | 2.6 ± 0.3 | 25.8 (22.5 – 29.7) | 26.1 |
| $F_{15}$ | 2.5 ± 0.3 | 9.3 (7.8 – 10.8) | 9.4 |
| $F_{16}$ | 2.9 ± 0.3 | 61.9 (54.6 – 71.1) | 62.5 |
| $F_{17}$ | 2.8 ± 0.4 | 75.3 (64.8 – 85.6) | 76.1 |
| $F_{18}$ | 2.9± 0.34 | 52.9(46.8 – 60.2) | 53.4 |
| $F_{19}$ | 3.2± 0.4 | 71.6(63.3 – 80.1) | 72.3 |
| $F_{20}$ | 2.6 ± 0.33 | 54.1(47.2 – 62.4) | 54.7 |

**Table 3: Cross resistance among spinosad and some pesticides used against pink bollworm resistance colony**

| Tested pesticides | Susceptible colony | | Resistant colony | | Resistance Ratios |
|---|---|---|---|---|---|
| | $LC_{50}$ and fiducial limits | Slope± SE | $LC_{50}$ and fiducial limits | Slope± SE | |
| Spinosad | 0.99 (0.84 –1.14) | 2.4 ± 0.33 | 54.1(47.2 –62.4) | 2.6 ± 0.33 | 54.7 |
| Buprofezin | 83.2(72.5 – 98.1) | 2.6± 0.33 | 89.1 (77.8 –105.3) | 2.7± 0.35 | 1.10 |
| Pyriproxyfen | 35.5(30.9 – 42.2) | 2.65± 0.34 | 39.9 (34.3 – 48.9) | 2.6± 0.34 | 1.12 |
| Carbaryl | 141.7(121.1-161.4) | 2.8 ± 0.4 | 102.3(86.9–115.2) | 3.9 ± 0.5 | 0.72 |
| Thiamethoxam | 10.5(8.9 – 12.1 0 | 2.4 ± 0.3 | 7.4(6.5 – 8.2 0 | 4.2 ± 0.5 | 0.70 |
| chlorpyrifos | 69.3(59.9 – 83.1 ) | 2.5 ± 0.3 | 16.9(5.2 – 25.4) | 1.3 ± 0.3 | 0.24 |
| L-cyhalothrin | 3.5 (3.1 – 3.9 0 | 3.4 ± 0.4 | 4.3(3.6 – 5.1 ) | 2.1 ± 0.3 | 1.22 |

**Table 4: Effect of piperonyl butoxide (PBO) on toxicity of spinosad**

| Treatments | $LC_{50}$ and fiducial limits | Slope ± SE | Synergism coefficient |
|---|---|---|---|
| Spinosad | 73.7(65.2 – 85.6) | 3.1 ± 0.4 | 3.8 |
| Spinosad and PBO | 19.2(11.8 – 25.00) | 2.1 ± 0.4 | |

**Table 5: Activity of acetyl choline esterase and total protein content in resistant and susceptible colonies of the pink bollworm, *P. gossypiella***

| Colonies | Activity of AChE ± SD (µ mole/ ml/ g tissue) | Total protein ± SD (g /dl/ g tissue) |
|---|---|---|
| Susceptible colony | [b]45.35±9.47 | [b]4.14±0.33 |
| Resistant colony | [a]91.23±12.56 | [a]17.79±2.95 |
| P value | 0.0071** | 0.0013** |
| LSD | 25.2 | 4.7 |

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