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Evaluations of *Isaria fumosorosea* Isolates against the Red Palm Weevil *Rhynchophorus ferrugineus* under Laboratory and Field Conditions

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ABSTRACT

Different concentrations of isolated (1) *Isaria fumosorosea* were*, studied against* different stages of red palm weevil (RPW) treated with the different fungi concentrations , the obtained LC50 249 x10^4, 239 X 10^4, 275 X 10^4, 268 X 10^4, 316 X 10^4 and 315 X 10^4 spores / ml for 2nd, 3rd, 4th, 5th, and adult ♀ and ♂ respectively., When RPW treated with isolate (2) of *I. fumosorosea* the corresponding LC50 obtained, 211 X 10^4, 286 X 10^4, 289 X 10^4, 297 X 10^4, 299X 10^4, 350 X 10^4, and 326 X 10^4 spores/ml., respectively. The third isolate of the fungus *I. fumosorosea*, affected on the target insect pest in all life cycle stages. Results indicated that the target insect pests were affected by *I. fumosorosea* isolate(1), isolated (2) *I. fumosorosea* and isolate (3) where the percentage of larval mortality were significantly increased to 97, 97% and 88% after treated with isolate *I. fumosorosea***,** respectively as compared to 1% in the control. At the same time the pupal mortality were affected by the three isolates treatments. The percentage of moth emerging were significantly decreased to 9% as compared to 100% in the control. The result show that the palm weight were significantly increased in El-Kassaseen (Ismailia) than in El-Esraa (Nobarya) to 5341± 40.30 kg/ Feddan as compared to 1981±80.54 kg/ Feddan in the control during season 2012. During season 2012 the yield loss obtained 59 and 62% in El-Esraa (Nobarya) and El-Kassaseen (Ismailia) which decreased to 28 and 27% in both corresponding regions the same results obtained during season 2013.

Key wards*: Isaria fumosorosea Rhynchophorus ferrugineus*, date palm.

Introduction

Rhynchophorus ferrugineus Olivier (Coeloptera: Curculionidae) is the most important pest of the date palm (*Phoenix dactylifera*) in the world. It is native to southern Asia and Melanesia, where it is a serious pest of coconuts (*Cocosnucifera*), and is a regulated and is a pest within the EU. Since the 1980s it has rapidly expanded its geographical range westwards. It reached Saudi Arabia and the United Arab Emirates in about 1985, spreading throughout the Middle East and into Egypt. In 1994 it was detected in Spain and in 1999 in Israel, Jordan and the Palestinian Authority Territories. It has since spread to the Balearic Islands (2006), Canary Islands (2005), Cyprus (2006), France (2006), Greece (2006), Italy (2004) and Turkey (2007). The two main palm species of concern in the Mediterranean region are date palm and Canary Island date palm (*P. canariensis*), the main crop and ornamental species, but it also attacks several other ornamental palms that are regularly imported into Britain, such as chusan palm (*Trachycarpus fortunei*). The European Commission has introduced emergency measures to prevent the further spread of *R. ferrugineus* within the community. Since it had been introduced to Egypt at1992 (Cox, 1993) thousands of healthy trees were damaged or lost (El-Sebaey, 2004). In infested plantations, yields have been estimated to drop from 10 tonnes to 0.7 tonnes per hectare (Singh and Rethinam, 2005). Furthermore, *R. ferrugineus* is a strong flyer. This increase the weevil's ability to disperse, colonize and breed at new sites (Murphy and Briscoe, 1999). However, longevity, activity and behavior of adult weevils are greatly affected by humidity. RPW controlled by chemical insecticides which pollute the environments .Unlike other insect pathogens, fungi infect the host by contact, penetrating the insect cuticle. The host can be infected both by direct treatment and by transmission of inoculum from treated insects or cadavers to untreated insects or to subsequent developmental stages *via* the new generation of spores (Lacey et al (2004), Qesada-Moraga et al (2004)). These unique characters make entomopathogenic fungi especially important for the control of concealed insects. In the case of *Rhynchophorus* spp., most of the life cycle passes within the tree trunk, making the pest inaccessible to direct-contact treatment. Adults are the only exposed stage and can be infected upon emergence. The questions remain of whether the treated adult will effectively convey the infection onwards and, if so, how and where it can best be treated.

Strains of *M. anisopliae* and *Paecilomyces fumosoroseus* were pathogenic to *C. capitata* adults under laboratory bioassay (Castillo *et al*., 2000). Konstantopoulou and Mazomenos (2005) reported that the usage of *B. bassiana* and *B. brongniartii* fungi were the most pathogenic to *C. capitata* causing 97.4 and 85.6%

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mortality. *M. anisopliae* cause a highly mortality rates to *C. capitata* and *B. oleae* adults and the rate of larval mortality was 85.2%. In Egypt, Mohamed (2009) reported that *Lecanicillim lecanii* and *M. anisopliae* fungi, and the interaction between *B. bassiana* and *M. anisopliae* fungi are suitable candidates to be used for control of *P. oleae.* P.f is a relatively new insecticide that is made up of two complex organic compounds, spinosyn A (right) and spinosyn D. These compounds are produced by certain microbes that were first discovered in soil found at an abandoned rum factory. Spinosad is a broad-spectrum, organic insecticide. It is, however, relatively non-toxic to mammals and beneficial insects. If used carefully only insects that actually eat something that has been treated, such as a leaf, are affected. This is different than a lot of other broad-spectrum insecticides that are toxic if the insect merely comes in contact with dry insecticide residues Qiao *et al*.(2012).

The present study aims to evaluate the pathogenicity of the entomopathogenic fungus, *Isaria fumosorosea* isolates against RPW under laboratory and field conditions. It is necessary to find alternative safety insecticides to reduce the heavy doses of chemical insecticides which is used for olive pests control.

Material and Methods

RPW collection: Random samples of RPW adults were collected from infested palm trees located at El-kasassen, Ismailia Governorate, Egypt. Insect rearing A RPW colony was established in the laboratory on sugarcane as both food and oviposition substrate, following Rahalkar *et al*., (1985). Adults were set to mate and oviposit in groups of at least five pairs placed on a substrate of moist sugar cane saw dust or on sugar cane logs. From the first larval instar to adult emergence, the RPWs were reared individually at 27–29º C. For egg harvesting, the adults of both sexes were kept on sugarcane sawdust. Eggs were collected every 2 days.

Source and Production of fungi:

Three isolates of the fungi *I. fumosorosea* from the dead RPW larvae. Isolated isolates were reproduced in the Microbiology Department, National Research Centre, Cairo, Egypt. They were primarily purified using the mono-spore technique. Then, propagated in Petri-dishes (9cm) on potato dextrose agar medium (PDAM) enriched with 1 %, peptone, 4 %. Glucose and 0.2% Yeast and incubated at 26 ºC. Seven days old cultures with well developed spores were harvested by washing with 10 ml sterilized water, then 3 drops of Tween-80 were added and completed to 100 ml with water. It was used as stock suspention and kept in a refrigerator at 4 ºC. From this stock (standared), dilutions with water were adjusted at the needed proposed concentrations. Large amounts of conidiospores, if needed, were produced by culturing the fungus on liquid medium in 1 L cell-culture glass bottles according to Rombach *et al* (1988) (modified by El-Husseini *et al*., 2004).

Isolation of the fungi:

The fungus *I. fumosorosea* was isolated from the diseased RPW insect pests and from Egyptian soil in el kassasin (Ismailia). Isolates were subcultured on nutrient PDA medium. Isolates were identified at National research Centre (NRC) Plant Pathology Department. The spores of *I. fumosorosea* were collected from agar surface of the fungus culture in 15cm diameter Petri-dish. Spore suspension in water + 0.1% Tween-80 was prepared. The strength of original culture was $1x10^8$ spore/ml. It was used as stock suspension and kept in a refrigerator at 4°C. From this stock, dilutions with water were adjusted at the needed proposed concentrations. Large amounts of conidiospores, if needed, were produced by culturing the fungus on liquid medium in 1 L cellculture glass bottles according to Rombach *et al*., (1988) and modified by El-Husseini *et al*., (2004).

Calculation of LC_{50}:

All fungal isolates concentrations of *I. fumosorosea* ranged from $1x10^2$ to $1x10^8$ spores/ml were prepared by 1-10 fold dilution from the main stock culture ($1x10^8$) and tested under controlled conditions (25±2ºC and 65±5% RH) against RPW larvae and adults. Ten 3-day-old weevils were collected in test tubes, immobilized on ice and carefully transferred to PDA dishes (9 cm diameter) containing the six fully developed fungal colonies. The weevils were allowed to walk on the fungal colonies for 5–10 min depending on fly mobility until the weevils collected spores on their body. The weevils were then removed from the Petri dishes and placed in small cages (10 cm x 10 cm x 10 cm). The same number of weevils treated similarly but with uninoculated PDA plates was used as controls. Solid diet and water were offered to weevils and kept under rearing conditions. Dead weevils were counted and removed from the cages daily for 21 days. Each treatment was replicated five times The percentages of mortality were calculated after seven days and corrected according to Abbott's formula (Abbott, 1925), while the LC_{50} value was calculated through Probit analysis according to Finney equation (Finney, 1971).

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Field experiments:

Esraa village- El-Nobaryia region and El-Kassaseen (Ismailia) during the two successive seasons 2012&2013 starting from the first of July till the end of October to evaluate the efficacy of the tested fungi against the target insect pests under field conditions. Three random patches of palm trees were selected, each comprised 12 trees (12 trees for *I. fumosorosea* isolate (1), *I. fumosorosea* isolate (2) , 12 trees for *I. fumosorosea* isolate (3) and 12 trees for control) to carry out the field experiment. *I. fumosorosea* was applied, each as a single treatment at the rate of $1x10^8$ spores/ml. Three applications were made at one week interval at the commencement of the experiment. Treatments were performed at the sunset with a ten litre sprayer. Percentage of infestation/sample was calculated after 20, 50, 90 and 120 days of the application. Each treatment was replicated four times. Four plots were treated with water as control. Random samples of leaves and fruits olives plants were weekly collected from each treatment and transferred to laboratory for examination. The infestation of RPW were estimated in each case.

After harvest, yield of each treatment was weighted as Kg/ Fadden. Yield loss was calculated according to the following equation:

$$\text{Yield loss} = \frac{\text{Potential yield- actual yield}}{\text{Potential yield}} \times 100$$

Potential yield was that yield obtained after *I. fumosorosea* isolate (1), treatment, which gave the best results among the tested pathogens, and was taken as a base for comparing with the other treatments

Bioassay Evaluation:

A- Larval treatment:

Isolates were tested selected against larvae in plastic boxes (35 X 20 X 30 mm) containing 2.5 g of moist sugar cane sawdust. Groups of five or six larvae were located in 9-cm petri dishes lined with 5-10 filter paper, sprayed with 2 ml of spore suspension containing $2 X 10^8$ spores/ ml. transferred to boxes (one larva per box) 5-10 min later. The control group was treated with an aqueous solution of 0.01% Triton X-100. The boxes were incubated at 27° C in darkness for 7-14 days. The larvae used for the bioassay weighed 60-200 mg. The bioassay was repeated three times and the results represented as were averaged according to Gindin *et al.* (2006). Mortality, malformation and emergence were recorded.

B- Eggs treatment:

The three isolates of *I. fumosorosea* were evaluated in this bioassay. Eggs for bioassay (1–4 days old) were obtained from egg laying cages. The eggs were placed singly in petri dishes each containing 3 g sugar cane sawdust Gindin *et al.* (2006). The petri dishes with eggs were incubated for 10 days at 27° C in darkness. Egg hatching and mortality of emerging larvae were observed during this period. The egg bioassay was repeated three times with three different batches of eggs (10–24 eggs per treatment of each test group).

C-Adult treatment:

Also designed according to Gindin *et al.* (2006). Subsequently, each treated or control weevil was placed in an individual box with 50 g of moist sugar cane sawdust, and incubated at 28° C under a 12:12 L:D regime for 2–7 weeks. Fresh substrate was added to the boxes every week. In order to evaluate the possibility of fungal transmission from surface-treated females to their progeny (eggs and larvae) during oviposition, 15 mated females were dusted individually with *I. fumosorosea* spores in 5 g of a solid rice-based formulation.

The control group comprised 15 untreated females which were incubated under the same regime up to completion of oviposition. Twice a week the sugar cane logs were replaced with new ones, and the old ones with eggs were incubated at 27° C in darkness for 14 days. The logs were then cut up and the eggs or hatched larvae were counted. The latter were placed in individual boxes with moist sugar cane sawdust and incubated for an additional 2 weeks to compare their survival with that of the controls. *Analysis of results Results:* Results of the bioassay were recorded as mortality percentages in larvae, pupae, hatching percentages of the eggs, malformed pupae and moths emergence. Student's t-test or one-way ANOVA was used to compare the effects of the experimental and control treatments on the eggs and larval mortality.

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Results and Discussions

Table 1 show that the LC50 of RPW first larval instars when treated with different concentrations of isolated *I. fumosorosea* isolate (1) were, 226X 10^4 spores/ml. when different stages of RPW treated with the different fungi concentrations , the LC50 obtained 249 x10^4, 239 X 10^4, 257 X 10^4, 268 X 10^4, 316 X 10^4 and 315 X 10^4 spores / ml for 2nd, 3rd, 4th, 5th, adult ♀ and ♂ respectively., (Table 1).

When RPW treated with isolate (2) of *I. fumosorosea* the corresponding LC50 obtained, were 211 X 10^4, 286 X 10^4, 289 X 10^4, 297 X 10^4 , 299X 10^4 , 350 X 10^4, and 326 X 10^4 spores/ml., for 2nd, 3rd, 4th, 5th, adult ♀ and ♂ respectively., (Table 2).

Table 3 show the effect of the third isolate of the fungus *I. fumosorosea* , which cleared that the fungus affected on the target insect pest in all life cycle stages .

The same results were obtained by Gindin *et al*. (2006) when found that fungi, *Metarhizium anisopliae* and *I. fumosorosea* strains of the former were found to be more virulent than those of the latter, achieving 100% larval mortality within 6.7 days. The same results obtained by Shaiju *et al* 2003, Gindin *et al.* (2006). The screening of fungi known to be pathogenic to RPW was aimed to evaluate the pathogenicity of *I. fumosorosea* three isolates to various development stages of RPW, and to develop a method for fungus treatment implementation.

The present investigation showed that the tested *I. fumosorosea* three isolates infect the larvae and fully completed their life cycles by forming conidiophores with conidia on RPW cadavers. The red pigmentation of larvae killed by *I. fumosorosea* indicates the presence of a red pigment, oospore in, produced by *Isaria* spp. (Gupta, *et al* 1995 and Vining, *et al* 1992).

Table 1: The insecticidal effect of fungi *Isaria fumosorosea* isolate (1) on *R. ferrugineus* .

Pathogen	LC50 (spores/ml)	Slope	Variance	95 % confidence limits
1st	226	1.1	2.3	200-344
2nd	249	0.2	1.4	222-288
3rd	239	1.1	0.3	214-288
4th	257	0.3	1.5	211-289
5th	268	1.2	1.3	217-300
Adults (♀)	316	2.0	1.8	299-323
Adults (♂)	315	0.1	1.1	289-332

Table 2: The insecticidal effect of fungi *Isaria fumosorosea* isolate (2) on *R. ferrugineus*.

Pathogen	LC50(spores/ml)	Slope	Variance	95 % confidence limits
1st	211 X 10^4	1.1	1.3	121-298
2nd	286 X 10^4	0.2	1.2	121-299
3rd	289 X 10^4	1.1	0.1	131-310
4th	297 X 10^4	0.3	1.4	140-319
5th	299X 10^4	1.2	1.3	209-318
Adults (♀)	350 X 10^4	2.0	1.4	270-361
Adults (♂)	326 X 10^4	0.1	1.1	289-341

Table 3: The insecticidal effect of fungi *Isaria fumosorosea* isolate (3) on *R. ferrugineus*

Pathogen	LC50(spores/ml)	Slope	Variance	95 % confidence limits
1st	231 X 10^4	1.1	1.3	221-297
2nd	275 X 10^4	0.2	1.2	210-299
3rd	289 X 10^4	1.1	0.1	210-310
4th	300 X 10^4	0.3	1.4	281-341
5th	311 X 10^4	1.2	1.3	290-349
Adults (♀)	354 X 10^4	2.0	1.4	301-348
Adults (♂)	328 X 10^4	0.1	1.1	249-352

The effect of the fungi *I. fumosorosea* were evaluated on the RPW eggs. Results showed that the isolated *I. fumosorosea* affected on the RPW eggs hatching. The percentage of eggs hatching were significantly decreased to zero % when the newly eggs treated with the isolated *I. fumosorosea* as compared to 100% in the control. The percentage of larval mortality were significantly increased to 100 as compared to 7% in the control (Table 4). The same results obtained by Gindin *et al.* (2006) who reported that, the pathogenicity of the two most virulent isolates of *M. anisopliae*, selected in the initial screening on larvae, was tested against *R. ferrugineus* eggs. Both isolates fungi killed the eggs quickly, without preliminary colonization on the egg surface. Gindin *et al.* (2006) reported that, all of the screened *M. anisopliae* strains exhibited pathogenicity to all development stages of RPW, causing up to 80–100% mortality of larvae and adult weevils under laboratory conditions. When eggs were exposed to sawdust previously sprayed with *M. anisopliae* spores, the total survival of both the eggs and the hatched larvae was reduced by a factor of approximately two to three, relative to control. Moreover, larvae developing in sprayed sawdust with fungal spores had a significantly reduced weight in comparison with larvae which emerged in the control treatment. The phenomenon could result from lower food intake by infected insects and/or from the energetic cost of confrontation with the infection. There are a

number of examples of reduction in food consumption in insects infected by pathogenic fungi (Ekesi, 2001,Tefera and Pringle (2003) and Soroker *et al.* (2005).

Table 5 show the effect of the three *I. fumosorosea* isolates tested against RPW under laboratory conditions. Results exhibited that the target insect pests were affected by *I. fumosorosea* isolate(1) and isolate (2) and isolate (3) of *I. fumosorosea* and the percentage of larval mortality were significantly increased to 97, 97% and 88% after treated with isolates of *I. fumosorosea*, respectively., as compared to 1% in the control. At the same time the pupal mortality were affected by the three treatments. The percentage of moth emerging were significantly decreased to 4% as compared to 100% in the control (Table 5). Magalhaes, *et al* 2001), found that, larvae of lower weight have lower and shorter life span. Gindin *et al.* (2006) reported that the direct effect on adult mortality

Table 4: Effect of the fungi *Isaria fumosorosea* isolate(2) on the Palm weevil *R. ferrugineus* eggs.

Eggs age	Eggs Hatching %						Larval mortality %					
	Isolates(1)		Isolates`(2)		Isolates(3)		Isolates(1)		Isolates(2)		Isolates(3)	
	T	C	T	C	T	C	T	C	T	C	T	C
Newly	0	100	1	99	11	00	3	100	2	100	4	100
Two days old	9	100	3	99	31	00	3	100	2	99	3	100
Three days old	12	99	10	100	11	99	3	100	3	100	2	99
Four days old	20	100	18	100	13	99	6	100	7	100	2	99

T. treated C. control (untreated)

Table 5: Effect of the fungi ***Isaria fumosorosea*** three isolates on the Palm weevil *R. ferrugineus* biology.

Fungal isolates	% Larval mortality	% of Malformed pupae	Pupal mortality	% of moth emerging
Control	1	0	0	100
L.l isolate(1)	97	61	59	10
L.l isolate(2)	97	50	84	4
L.l isolate(3)	88	55	75	7

Table 6 and 7 show the effect of the three isolated fungi of *I. fumosorosea* on RPW in two Egyptian regions differ in climatic conditions in El-Kassaseen (Ismailia) clay soil and El-Esraa (Nobarya) sandy soil . The result showed that the palm weight were significantly increased in El-Kassaseen (Ismailia) than in El-Esraa (Nobarya) to 5341± 40.30 kg/ Feddan as compared to 1981±80.54 kg/ Feddan during season 2012. During season 2012 the yield loss obtained 59 and 62% in El-Esraa (Nobarya) and El-Kassaseen (Ismailia)which decreased to 28 and 27% in both corresponding regions (Table 6). The same results obtained during season 2013.

The yield weight were significantly increased to 5875± 34.35 Kg/feddan in El-Esraa (Nobarya)as compared to 1999± 36.81Kg/feddan in the control. In El-Kassaseen (Ismailia), the yield were significantly increased to 5997±65.31Kg/feddan as compared to 1791±80.44Kg/feddan in the control. The yield loss ranged between 70 and 35% (Table 7).

Gindin *et al.* (2006) Despite the observed susceptibility of all the development stages of the RPW to the entomopathogenic fungi under laboratory conditions, the practicability of achieving efficient control of RPW in the field seems problematic. The field efficacy of entomopathogenic fungi toward various pests depends on many factors, often related to the behavior of the insect host in its natural habitat. The soil is the natural habitat of fungi and, since the RPW pupae occasionally inhabit the soil, it is theoretically possible to infect them with fungal spores by soil treatment. The spraying of palms and of large areas between them to ensure contact between free living adult RPW and fungal spores also presents a difficulty, because of the large fungal inoculum needed. The best strategy would be to treat only selected areas that are especially likely to attract adults. Adult weevils are usually cryptic, taking refuge between petioles and offshoot bases. They are highly attracted to wounds in palm trees, e.g. that are infected during vegetative production practices that include the removal of offshoots (Furlong and Pell (2001)). These areas are often the most attractive sites to females for oviposition and, therefore, may be the best candidates for treatment with a dry fungal formulation. The possibility of infecting the Rhynchophorus adults by this method was discovered by chance, after application of a rice-based formulation of *M. anisopliae* against the *Scarabaeid Scapanes*aus tralis on young palms in New Guinea (Prior,.and Arura, (1985). The treatment of frond axils with this formulation caused infection not only to the target pest but also infected some incidental infection on *R. bilineatus*. The high mortality of adults to dry spores of a selected isolate indicates that there was proper contact between fungi and ovipositing females. Hydrophobicity of fungal spores could play a significant role in the success of the dry rice formulation. The attachment of a fungal spore to the cuticle surface is the initial and thus a crucial event in the establishment of mycosis (Boucias, *et al* 1998). The spores of *M. anisopliae* possess an outer layer composed of interwoven fascicles of *hydrophobic rodlets*, which provided the adhesion to the insect cuticle due to non-specific hydrophobic forces (Boucias, *et al* 1998). Thus, similar to the natural infection process, the application of dry spores could provide better adhesion relative to application of aquatic spore suspension. Ideally, we would like to use artificially inoculated females as vectors of infection to their progeny via egg contamination during

oviposition. However, in the present study we were unable to prove that such infection transfer occurred. We believe that the lack of success was due mainly to insufficient inoculum transferred by females to eggs. It is possible that this problem is connected with spore hydrophobicity. This character may interfere with fungus transfer from female hydrophobic surface to the egg under humid conditions during oviposition into the plant tissue. The mechanical transfer of fungal spores between the oviposition sites and even further into the oviposition tunnels would be possible, provided that a suitable fungus formulation could be developed. In light of the high susceptibility of eggs and larvae to fungal infection, we assume that in such a case the contamination of eggs and of the larval habitat would add to the effectiveness of this pest control method.

Table 6: Weight of harvested palm and percentage of yield loss after treatment with the fungi ***Isaria fumosorosea*** against *RPW* during seasons 2012 in two different regions.

Treatments	El-Esraa (Nobarya)		El-Kassaseen (Ismailia)	
	Weight palm (Kg/feddan)	% yield loss	Weight palm (Kg/feddan)	% yield loss
Control	2000± 36.82	59	1981±80.54	62
L.l Isolate(1)	4998± 41.20	-	5341± 40.30	-
L.l Isolate(2)	4560± 34.31	8	4997±65.32	6
L.l . Isolate(3)	3541± 42.57	28	3879±69.33	27
F values	31.02		32.31	
LSD 5%	92		90	

Table 7: Weight of harvested palm and percentage of yield loss after treatment with the fungi *Isaria fumosorosea* against RPW during 2013 in two different regions.

Treatments	El-Esraa (Nobarya)		El-Kassaseen (Ismailia)	
	Weight palm (Kg/feddan)	% yield loss	Weight palm (Kg/feddan)	% yield loss
Control	1999± 36.81	65	1791±80.44	70
*L.l*Isolate(1)	5875± 34.35	-	5997±65.31	-
L.l . Isolate(2)	3113± 42.50	47	3993± 42.50	33
L.l Isolate(3)	3473± 42.50	40	3892±69.73	35
F values	33.02		30.30	
LSD 5%	84		80	

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