Pathogenicity of *Helicoverpa armigera* Nucleopolyhedro virus (*Hear*NPV) and *Lecanicillium* sp. against *Helicoverpa armigera*

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ABSTRACT

Helicoverpa armigera (Hubner) is an important pest of corn crops. *Lecanicillium* sp. and *Helicoverpa armigera* Nucleopolyhedro virus (*Hear*NPV) are biological control agents that are safe for the environment. This study observed pathogenicity of *Hear*NPV and *Lecanicillium* sp. against eggs and larvae *H. armigera*. The results showed that *Hear*NPV application was not able to infect eggs, but the mortality effects of newly hatched larvae and second instars larvae at density of 10^7 was 100% and 90% respectively, while in mixed applications *Lecanicillium* sp. and *Hear*NPV on eggs at 10^7 caused 17.5%, and eventually died were 100%. At 10^7 concentration with mixure of application (*Lecanicillium* sp. and *Hear*NPV) mortality was only 26.25%. The value of LT_{50} in *Hear*NPV application against *H. armigera* larvae was 2.03 days faster than that of mixed applications on 3.23 days. A conclusion statement *Hear*NPV was more effective against *H. armigera* than mixed application (*Hear*NPV and *Lecanicillium* sp.)

Keywords: Egg, *H. armigera*, larvae, lethal time, mortality.

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INTRODUCTION

Corn has a strategic role in the national economy because of its multipurpose function as food, feed, and industrial raw materials (Aldillah, 2017). As foodstuff, corn is the second source of carbohydrate after rice that plays a role in supporting food security. One type of corn widely consumed is sweet corn (*Zea mays saccharata*) because it has a sweeter taste than regular corn (Widiani *et al.*, 2017). *Helicoverpa armigera* (Noctuidae) is a major pest in corn crops, widely spread from Europe, Asia, Africa and Australasia (Anderson, 2016) to south and central America, north America and Mexico (Kriticos *et al.*, 2015). It affects corn, cotton, tomatoes, tobacco, sunflower, peas and cut flowers (Fatma and Pathak, 2012). *Helicoverpa armigera* cause large yield loss and deterioration in the quality of corn plants. Based on crop damage intensity reaching 80-90%, the most effective insecticide for controlling *H. armigera* in the field is indoxacarb (Kim *et al.*, 2018).

The continuous use of insecticides causes *H. armigera* to become resistant. So other alternatives have to be found out. In Indonesia impact of *Hear*NPV against *H. armigera* larvae was studied by many scientists (Tenrirawe, 2011, Yasin *et al.*, 2005, Bedjo, 2012). At concentration N. rileyi of 2x108 conidia/ml, mortality of *H. armigera* larvae was 86.67%, and 90% at application *B. bassiana*, 10 days after treatment. HearNPV at concentration of 10^6 PIB/ml caused 100% mortality of larvae, but the mixed application decreased mortality of *H. armigera* larvae. On the mixed application of *N. rileyi* at concentration $2x10^8$ conidia/ml + HearNPV 10^6 PIB/ml, mortality was 90% and 93.33% at mixed application B. bassiana $2x10^8$ conidia/ml + HearNPV 10^6 PIB/ml (Gundannavar *et al.*, 2004). However,

information about the impact of *Lecanicillium* sp. and *Hear*NPV mixture was not available in the literature. The results of the earlier chapter show that *H. armigera* larvae mortality due to *Lecanicillium* sp. treatment at 10^7 conidia/ml density was 41.25%, with LT_{50} of *Lecanicillium* sp. was 7.12 days and 1.7 x 10^6 conidia/ml value of LC_{50} (Ginting, 2018). The use of *Hear*NPV as a biological agent has disadvantages because it has a specific host. In addition, it takes long time to kill target pests ranging from 5 to 12 days after being infected (Vega and Kaya 2012). This weakness is the general character of bioinsecticides, not only viruses, but also fungi. Based on this, pathogenicity testing *Lecanicillium* sp. and *Hear*NPV against *H. armigera* has to be done. This study observed pathogenicity of *Hear*NPV and *Lecanicillium* sp. against eggs and larvae *H. armigera.*

METRIALS AND METHODS

Preparation of *Hear*NPV and *Lecanicillium* sp.

Hear NPV and *Lecanicillium* sp. were collected from the Insect Pathology Laboratory of Plant Protection Department, Faculty of Agriculture, Bogor Agricultural University. The source of *Hear* NPV from a cotton plantation in Gowa-South Sulawesi Province that has similar identification with the Brazilian *Heze* NPV isolate (Diyasti, 2016).

Propagation and Purification of Virus Particles

Methods for purification of viral particles was carried out according to the methodology of Shepard (1994). One hundred larvae that have died infected with the viruswere crushed with a mortar in 0.1% sodium dodecyl sulfate (SDS) solution, and filtered with a 100 mesh gauze. Then the suspension was put into eppendorf micro tube and centrifuged at 3500 rpm for 30 minutes. The resultant pellet was mixed again in 0.1% SDS solution and centrifuged again at 13.000 rpm for 90 minute in 40-60% (w/v) continuous sucrose gradient at 4 °C. The viral layer retained by sucrose solution was taken with a pipette and dissolved in 10 ml of 0.1% SDS solution. The treatment in sucrose gadient was done twice to obtain pure virus particles, then the pure viral particles were dissolved in sterile water. The concentration of polihedra contained in the virus suspension that had been washed was calculated using a hemocytometer, under a light microscope with an enlargement of 40 x.

*Hear*NPV against egg and larvae of *H. armigera*

The concentrations suspension of *Hear*NPV polyhedra used were 10^5, 10^6, 10^7 PIBs/ml and control (sterile water). The eggs insect tests were done with eggs one day old with 20 eggs per replication. The insect larvae test used second instar with 20 larvae per replication and repeated four times for each treatment. Methods of application of the *Hear*NPV against the egg was carried out according the methodology of Tefera and Pringle (2003). The *Hear*NPV application to the egg was done by immersing the egg in polyhedral suspension for 60 seconds, then the eggs were moved into a petri dish and were observed until the eggs hatched. The observed variables were the percentage of hatching eggs and the percentage of instar first larvae infected by the fungus. The test insect larvae used were second instar, with 20 larvae per replication and repeated four times each treatment. The virus application against the larvae was carried out according to the methodology of Hunter Fujita *et al.,* 1998. Artificial with size of 1x1x0.5 cm feed dipped in the viral suspension for ten seconds and dried (± 5 minutes), then given as feed for *H. armigera* larvae. Larval mortality was observed after the *Hear*NPV application daily until seven days.

Mixture *Hear*NPV with *Lecanicillium* sp. against eggs and larvae *H. armigera*.

The mixture concentrations of *Hear*NPV with *Lecanicillium* sp. used were *Hear*NPV: 10^5, 10^6, 10^7 PIBs/ml + *Lecanicillium* sp.: 10^5, 10^6, ^{107} conidia/ml and control. The test eggs insect used were one day old with 20 eggs per replication. The test insect larvae used second instar with 20 larvae per replication, each treatment repeated four times. The application done by immersing the egg in a conidia suspension for 60 seconds, then the eggs were

moved into a petri dish and observed until the eggs hatched. The observed variables were the percentage of hatching eggs and the percentage of first instar larvae infected by the pathogen.

Application of suspension mixture of *Lecanicillium* sp. and *Hear*NPV in larvae were carried out separately at the same time. The *Hear*NPV application to larvae was carried out using a method of feed contamination (Hunter-Fujita *et al.,* 1998) which was carried out by artificial feed with a size 1x1x0.5 cm dipped in virus suspension according to concentration of each treatment for ten seconds and dried (± 5 minutes) and then given as feed for *H. armigera* larvae. Fungus application on larvae was done by spraying larvae with suspension in each treatment according to the methodology of Tefera and Pringle (2003). Larval mortality was observed every day until seven days after application. Data Analysis The experimental data ware analyzed using SPSS programversion 16.1, In case of any difference between treatments, data analysis was then continued with Duncan Multiple Range Test in level α 0.05. Determination of LT25, 50, 75 values by probit analysis was done according to Finney, 1971 by using SAS program version 6.12.

RESULTS

*Hear*NPV to eggs and first instar larvae of *H. armigera.*

The results of the pathogenicity test on eggs indicated that *Hear*NPV cannot inhibit egg hatching. *H. armigera* eggs which applied with *Hear*NPV suspension in all treatments were still able to hatch 100% (Table 1), but the application of the virus affected the survival of the newly hatched first instar larvae. The highest mortality of new hatched instar larvae at concentration of 10^7 PIBs/ml was not significantly different from that at the concentrations of 10^5 and 10^6 PIBs/ml. The mortality of first larvae is caused by the newly hatched larvae eating the eggshells so that the *Hear*NPV attached to the egg shelis consumed by the larvae.

*Hear*NPV to Second Instar Larvae *H. armigera.*

*Hear*NPV which we used as bioinsecticide for *H.armigera* control was very effective. This can be seen from the high percentage of mortality of second instar larvae that die in each treatment. The highest mortality of second instar larvae occurring at concentration 10^7 (PIBs/ml) was 90%, but not significantly different from the treatment of 10^5 and 10^6 (PIBs/ml) treatment (Figure 1 and Table 1). The use of high concentrations causes more polyhedra consumed by *H. armigera* larvae, thus accelerating the process of larval death.

Table 1. Effect of *Hear* NPV at various concentrations against egg, newly hatched *H. armigera* eggs or first instar and larvae mortality second instar in the third day after application

Concentration (PIBs/ml)	*Hear* NPV		
	Egg mortality (%) ± SD	Larval mortality first instar (%) ± SD	Larval mortality second instar (%) ± SD
0 (Control)	0 ± 0.00 a	00.00 ± 0.00 a	00.00 ± 0.00 a
10^5	0 ± 0.00 a	97.50 ± 1.15 b	73.75 ± 3.86 b
10^6	0 ± 0.00 a	97.50 ± 0.57 b	83.75 ± 3.20 b
10^7	0 ± 0.00 a	100.00 ± 0. 00 b	90.00 ± 1.41 b

The numbers followed by different letters in the same column are not significantly different according to the Duncan test at a real 5% level.

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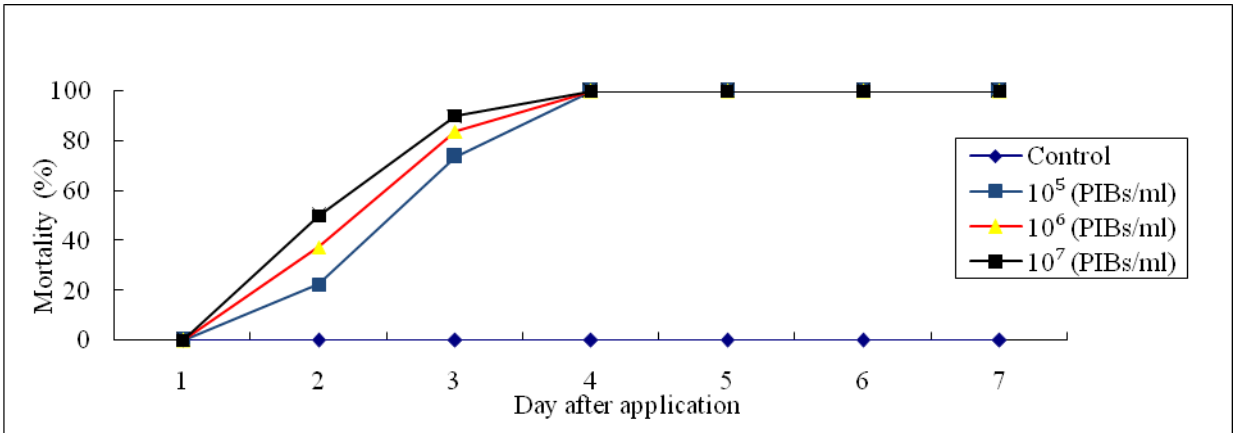

Figure 1. Mortality of second instar larvae due to *Hear*NPV treatment at various concentrations

Lecanicillium* sp. and *Hear*NPV against eggs and newly hatched first instar larvae of *H. armigera

Mixed pathogenicity test results of *Lecanicillium* sp. and *Hear*NPV against *H. armigera* eggs showed that a mixture of pathogens was able to inhibit *H. armigera* hatching eggs. The highest egg mortality at density of 10^7 conidia/ml + 10^7 PIBs/ml was 17.50% and the lowest in the 10^5 conidia/ml + 10^5 PIBs/ml treatment was 1.30% (Table 2). The mortality of the survival of newly hatched first instar larvae increases with the increasing concentration of pathogens applied to *H. armigera* eggs. The highest larval mortality was the same as in egg treatment. At the treatment of 10^7 conidia/ml + 10^7 PIBs/ml was 100%, and 95% at 10^5 conidia/ml+10^5 PIBs/ml treatment.

Mixed applications (*Lecanicillium* sp. and *Hear*NPV) for the survival of newly hatched first instar larvae was lower than the *Hear* NPV application. Mortality of first instar larvae on three days, at *Hear*NPV with concentration 10^5 PIBs/ml was 97.5%, while in mixed applications it was 95%. *Lecanicillium* sp. and *Hear*NPV against instar larvae of *H. armigera.* The mixed (*Lecanicillium* sp. and *Hear*NPV) can cause mortality in second instar *H. armigera* larvae, but the mortality of larvae in this treatment was lower compared to that is *Hear*NPV. The mortality of the second instar larvae on three days highest occurred in the mixture of *Lecanicillium* sp. at concentration 10^7 conidia/ml + HearNPV 10^7 PIBs /ml was 26.25% and the lowest in treatment 10^5 was 10% (Table 2 and Figure 2); while the *Hear* NPV application at concentration 10^7 PIBs /ml treatment was 90%.

Table 2. Effect of mixture (*Lecanicillium*sp. and *Hear*NPV) at various concentrations against egg, newly hatched *H. armigera* eggs or first instar and larvae mortality second instar in the third day after application.

Concentration (PIBs/ml)	*Lecanicillium* sp. and *Hear*NPV		
	Egg mortality (%) ± SD	Larval mortality first instar (%) ± SD	Larval mortality second instar (%) ± SD
0 (Control)	0.0 ± 0.00a	00.00 ± 0.00 a	00.00 ± 0.00 a
10^5	1.3 ± 0.50a	95.00 ± 0.00 b	10.00 ± 2.16 ab
10^6	7.5 ± 1.29ab	96.25 ± 0.95 b	17.50 ± 2.64 bc
10^7	17.5 ± 3.69b	100.00 ± 0.00 c	26.25 ± 0.95 c

The numbers followed by different letters in the same column are not significantly different according to the Duncan test at a real 5% level.

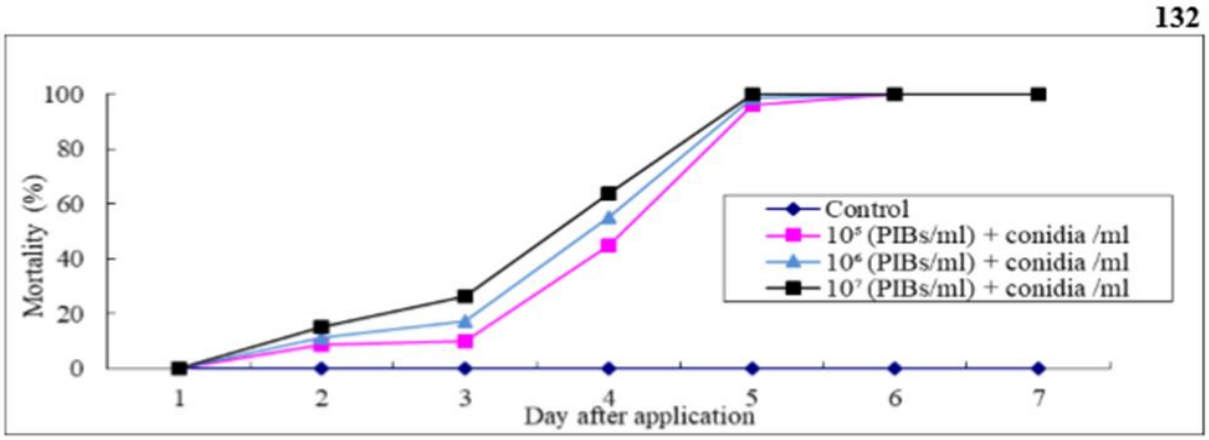

Figure 2. Mortality of second instars larvae due to mixed treatment (*Lecanicillium* sp. and *Hear* NPV) at various concentrations.

The value of LT_{50} in *Hear*NPV application against *H. armigera* larvae is lower than that of mixed applications. LT_{50} of *Hear*NPV was 2.03 days and LT_{50} of mixed applications was 3.23 days and the longest time of death occurred in the fungus *Lecanicillium* sp. (Table 3).

Regression equation between density log and *Hear*NPV probit value, *Lecanicillium* sp. and the mixed (*Hear*NPV and *Lecanicillium* sp.) for *H. armigera* mortality on the three day are shown in Figure 3.

Table 3. The value of LT results from the analysis of all pathogenic probits at concentrations 10^7 against second instars larvae

Isolate (concentration)	LT_{25}	LT_{50}	LT_{75}
	Day		
*Hear*NPV (10^7 PIBs/ml)	1.70	2.03	2.44
*Hear*NPV and *Lecanicillium*sp. (10^7 conidia/ml + 10^7PIBs/ml)	2.62	3.23	3.98
*Lecanicillium*sp. (10^7 conidia/ml)	3.95	7.12	12.82

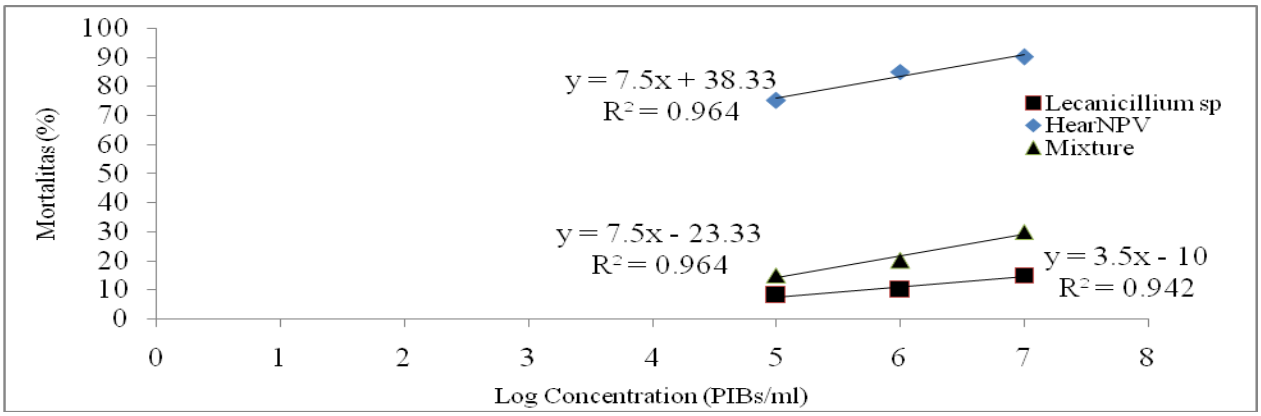

Figure 3. Mortality of the LC (lethal concentration) *Hear* NPV, *Lecanicillium* sp. and mixture (*Lecanicillium* sp.and *Hear*NPV) against second instars larvae of *H. armigera* at 10^7 PIBs /ml density.

The positive relationship between concentration and mortality indicates that high concentrations that increase *H. armigera* mortality with the regression equation of each pathogen show a relationship between logarithm concentration and probit percentage of larval mortality. The positive slope value shows a relationship that is directly proportional, meaning that the higher the X value, the greater the Y value. The *Hear*NPV virus, *Lecanicillium* sp. and the mixed (*Hear* NPV and *Lecanicillium* sp.) had value of R^2 = 0.9, meaning that the correlation coefficient between variables X and Y was very strong, so that infections caused by pathogens affect the mortality of *H. armigera* larvae. This was possible in controlled environmental conditions such as in research laboratories.

DISCUSSION

The results of pathogenicity test of the *Hear NPV* on *H. armigera* eggs showed that *Hear* NPV was unable to infect eggs *H.armigera*. It was caused by *Hear*NPV as stomach poison, moreover the virus could not replicate the eggs because of the dry egg condition. Eggs treated with a mixture of *Lecanicilium* sp. and *Hear* NPV can cause egg mortality by 17.50% (Table 1). Inhibition of egg hatching occurs due to the mechanism of action of *Lecanicilium* sp. The fungus was able to germinate due to stimulation of environmental factors (humidity and temperature). Agustin (2014) reported that *L. lecanii* was able to infect *O. furnacalis* eggs in the laboratory and the egg mortality was 100%. The results of *Hear*NPV application against *H. armigera* showed that the mortality rate of first instar larvae was 100%, and 90% in the second instar at 10^7 PIBs/ml treatment. This indicates that the pathogen was very effective against *H. armigera*. In a mixture of *Lecanicilium* sp. and *Hear*NPV mortality of newly hatched first instar larvae was 100% at the concentration of *Lecanicillium* sp. 10^7 conidia/ml + *Hear*NPV 10^7 PIBs /ml, and mortality in the second instar larvae was lower than in *Hear*NPV application 26.25% (Table 2). Additive effects on mixed applications did not occur in viruses, when compared to the than in *Hear*NPV application 26.25% (Table 2). Additive effects on mixed applications did not occur in viruses, when compared to the *Hear*NPV application. The same thing was reported by Gundannavar *et al.*, 2004, *Hear* NPV isolates at concentration of 10^6 PIB/ml caused 100% mortality of *H. armigera* larvae whereas, the mixture application decreased mortality of *H. armigera* larvae. *N. rileyi* mixed application at concentration of $2x10^8$ conidia / ml + *Hear*NPV 10^6 PIB/ml caused mortality by 90%, and B. bassiana at $2x10^8$ conidia/ml + *Hear*NPV 10^6 PIB/ml by 93.33%, 10 days after application. Yasin *et al.*, (2005) reported that the treatment of *Hear* NPV at concentrations 10^6 PIBs/ml until 10^8 PIBs/ml caused 90.60% mortality of *H. armigera* larvae. It is a very effective was to control *H. armigera* larvae. Another possibility was the treatment of fungi on the body of the larvae resulting in decreased feeding activity so that the amount of virus consumed was reduced, thereby reducing larval mortality. The abundant PIBs eaten makes the larvae die quickly because more virions will damage the mesenteron larval (Gupta *et al.*, 2007). The death of first instar larvae was caused by these coming out of the egg eating eggshells so that PIBs and conidia attached to the eggshell were also eaten by larvae. As a result infection occurs through the digestive tract. Other possibilities of infection also occur when neonate larvae come out of their shells, neonates eating eggshells and conidia attached to eggshells derived from aplication of the conidia suspension consumed by newly released larvae so that infection occurs through the digestive tract. The same thing was reported by Jayaraj in Sigsgaard *et al.*, 2002. That *H. armigera* neonates eat eggshellsis reported by them. *H. armigera* larvae attacked by *Hear*NPV showed symptoms of reduced eating activity, sluggish movements, integument getting darker, body becoming weak and flabby, and release of cloudy and smelly fluid from the body. Insects infected with the virus show symptoms of morphological and physiological behavior. After 2-5 days the larvae eat parts of plants that contain polyhedral (Vega and Kaya,

2012). Entomopathogenic fungi coused mortality of the host by digesting the tissue as a source of nutrients and producing toxins that play a role in killing the host. Vey *et al.*, 2001 suggested that *L.lecanii* produces several types of toxins among others dipicolinic acid, hydroxycarboxylic acid and cyclosporine at different levels. Cyclosporin toxin can cause interference with the function of the hemolymph and insect nucleus, resulting in swelling accompanied by hardening of infected insects. After the larvae die, hyphae will grow on the surface of the larvae and will spread to find new hosts or experience dormancy (Vey *et al.*, 2001).

CONCLUSION

*Hear*NPV was more effective against *H. armigera* than mixed application (*Hear*NPV and *Lecanicillium* sp.) *Hear*NPV application was not able to infect eggs, but caused 100% mortality of newly hatched larvae at a density of 10^7 and 90% mortality of second instar larvae at the same density; while in mixed applications of *Lecanicillium* sp. and *Hear*NPV at a density of 10^7 the mortality of eggs was 17.5%, and from hatched eggs, all the emerging larvae eventually died. At concentration of 10^7 with mixed application (*Lecanicillium* sp. and *Hear*NPV) the mortality reached 26.25% on three days. The value of LT_{50} in *Hear*NPV application against *H. armigera* larvae is lower than that of mixed applications.In LT_{50} of *Hear*NPV it was 2.03 days and in LT_{50} of mixed applications it was 3.23 days.

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