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Original article (Orijinal araştırma)

Development of densovirus-based bioinsecticide (Tenebriokiller) for use in the control of mealworm, *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae)[1]

Un kurdu, *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae)'un kontrolünde kullanılmak üzere densovirüs bazlı biyoinsektisitin (Tenebriokiller) geliştirilmesi

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Abstract

Tenebrio molitor L., 1758 (Coleoptera: Tenebrionidae) is one of the major storage pests affecting a large proportion of pasta, flour, starch, and rice. Densoviruses are biocontrol agents with a single-stranded DNA genome that is enveloped and very small in size. In this study, crude TmDNV-TR was formulated using bovine gelatin, and the formulation called Tenebriokiller was prepared in Türkiye. The dose of virus in the formulation was determined to be $2.8x10^8$ copies/mL^{-1}. Five different doses were used ($2.8x10^8$, $2.8x10^7$, $2.8x10^6$, $2.8x10^5$, and $2.8x10^4$ copies/mL^{-1}) against 10th instar *T. molitor* larvae. The mortality was detected 100% (LD_{50}: 4 $x10^6$ copies/mL^{-1}, LT_{50}: 6 day, LD_{95}: 2.2 $x10^8$ copies/mL^{-1} and LT_{95}: 9 day) by day 14. The stability of the crude virus and Tenebriokiller was tested at different storage temperatures (4 and 25°C) over various periods (3 and 6 months). Accordingly, the mortality rate of the fresh formulation was 100% as in the previous step, while at 4°C the mortality rate at the end of 3 and 6 months remained 100%, and at 25°C, the mortality rate at the end of 3 and 6 months was 98% and 95% respectively, at the end of day 14. Preliminary pathogenicity tests in the laboratory showed that the virus was highly lethal to the target organism. The virus was therefore formulated to maintain its stability during storage, and a prototype formulation was developed.

Keywords: Biocontrol, densovirus, *Tenebrio molitor*, virus formulation, virus stabilization

Öz

Tenebrio molitor L., 1758 (Coleoptera: Tenebrionidae), makarna, un, nişasta ve pirincin büyük bir kısmını etkileyen önemli depo zararlılarından biridir. Densovirüsler, zarflı ve çok küçük boyutlu tek zincirli bir DNA genomuna sahip biyolojik kontrol ajanlarıdır. Bu çalışmada, sığır jelatini kullanarak ham TmDNV-TR formüle edilmiş ve Tenebriokiller adı verilen formülasyon Türkiye'de hazırlanmıştır. Formülasyondaki virüs dozunun $2.8x10^8$ kopya/mL^{-1} olduğu belirlenmiştir. Beş farklı doz ($2.8x10^8$, $2.8x10^7$, $2.8x10^6$, $2.8x10^5$ ve $2.8x10^4$ kopya/mL^{-1}) 10. dönem *T. molitor* larvalarına uygulanmıştır. Ölüm oranı 14. günün sonunda %100 (LD_{50}: 4 $x10^6$ kopya/mL^{-1}, LT_{50}: 6 gün, LD_{95}: 2.2 $x10^8$ kopya/mL^{-1} ve LT_{95}: 9 gün) olarak belirlenmiştir. Ham virüsün ve Tenebriokiller'in stabilitesi farklı depolama sıcaklıklarında (4 ve 25°C) çeşitli sürelerde (3 ve 6 ay) test edilmiştir. Buna göre, taze formülasyonun ölüm oranı bir önceki adımda olduğu gibi %100 iken, 4°C'de 3 ve 6 ayın sonunda ölüm oranı %100, 25°C'de ise 3 ve 6 ayın sonunda ölüm oranı sırasıyla %98 ve %95 olarak belirlenmiştir. Laboratuvardaki ön patojenite testleri virüsün hedef organizma için oldukça öldürücü olduğunu göstermiştir. Bu nedenle virüs, depolama sırasında stabilitesini koruyacak şekilde formüle edilerek, bir prototip formülasyon üretilmiştir.

Anahtar sözcükler: Biyolojik mücadele, densovirüs, *Tenebrio molitor*, virüs formülasyonu, virüs kararlılığı

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Introduction

Entomopathogenic viruses stand out as prominent biological control agents owing to their narrow host spectrum, low production costs in the insect body, and environmental safety (Aş & Eroğlu, 2025). Entomopathogenic viruses that can cause oral infections are being recognized as ecologically sustainable alternatives for chemical insecticides in pest management (Deka et al., 2021). Their inability to adversely affect non-target organisms and their role in maintaining ecological balance offer notable advantages in the context of biological control strategies (Cory, 2003). In addition, their negligible environmental footprint aligns with sustainable agriculture principles (Thapa et al., 2020).

Densoviruses, which belong to the subfamily Densovirinae of the family Parvoviridae, are small, non-enveloped viruses with linear, single-stranded DNA genomes (Cotmore et al., 2019). These viruses have been isolated from a wide range of insect orders, including Dictyoptera, Diptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera, and Orthoptera (Aş & Eroğlu, 2025). Although densoviruses were first identified nearly six decades ago, their potential as biological control agents was not seriously explored until it was conclusively demonstrated that they do not infect vertebrates (Johnson & Rasgon, 2018). Subsequent *in vitro* and *in vivo* studies in mammals confirmed this host restriction, leading to a surge of research into their utility in pest control programmes over the last twenty years (Batool et al., 2022). Densoviruses offer several advantages, including their specificity to invertebrates, the ability to replicate via oral infection, and high environmental stability (Pigeyre et al., 2019; Penzes et al., 2024). To date, one commercial densovirus-based product (Biokiller, China) has been produced. This product, which contains a densovirus (PfDNV) isolated from *Periplaneta fuliginosa*, was developed in gel form for cockroach control and is commercially sold under the name Biokiller Cockroach Killer Bait Gel (biological, WDLZ-BL-5) (Dong et al., 2012).

Tenebrio molitor L., 1758 (Coleoptera: Tenebrionidae), which damages pasta, flour, starch, and rice that are very important in terms of both commercial and nutritional value, reproduces rapidly and spreads quickly to products in storage (Kavallieratos et al., 2019). Thus, it causes economic losses and contamination of products (Plata-Rueda et al., 2017). To cope with this problem, producers often apply chemical pesticides to their storage facilities. As chemical pesticides have carcinogenic effects, they both harm the person applying them during application and threaten the health of consumers by leaving residues on food. In addition, chemical pesticides cause the development of resistance in insects (Talukder, 2009).

This study aims to evaluate the stability under storage conditions of a densovirus isolate that has the potential to be used as a biological control material for the biological control of *T. molitor*, which causes economic losses in stored produce, by developing a formulation. Preliminary pathogenicity tests in the laboratory showed that the virus was highly lethal to the target organism. The virus was therefore formulated to maintain its stability during storage, and a prototype formulation was produced.

Materials and Methods

Insect rearing

Tenebrio molitor larvae used in the experiments were obtained from a local pet shop. Species identification was confirmed by examining the larvae under a binocular microscope, following the method described by Brendell (1975). The larvae were housed in air-permeable plastic boxes (9 × 16 × 5 cm) and maintained in a climate chamber set at 26°C, 65% relative humidity, and a 12:12 h light/dark photoperiod. New generations were reared by feeding on cracked wheat to be used in the experiments (Ribeiro et al., 2018).

Dose-response test of crude virus

The virus which was previously detected and purified is available in our laboratory stocks (Aş et al., 2025). The virus dose was determined by real-time PCR according to La Fauce et al. (2007). Five different doses ($4x10^8$, $4x10^7$, $4x10^6$, $4x10^5$, and $4x10^4$ copies/mL^{-1}) were prepared by serial dilution of the stock virus dose. For each experiment, 30 10^{th} instar medium larvae, were selected and individually placed in

plastic containers (7.5x5x2.5 cm) where they were starved for 16 h. The 10 mL pure crude virus was sprayed on the 100-gram cracked wheat. The food of the larvae in the control group was sprayed with 10 mL dH_2O using a hand held mini sprayer. The insects were placed in containers once the food had dried. The insects were kept in Biotest containers in a climatic chamber (26°C, 12:12 h light: dark, 65% humidity), and deaths were checked daily.

Virus purification

Virus purification was performed on *T. molitor* cadavers that had died as a result of showing densovirus symptoms (blackening of the body) as a result of the previous step experiment. These larvae were placed in test tubes and ground in a tissue homogenizer for 45 min. They were filtered 3 times through cheesecloth to remove cellular debris and through a 0.22 µm filter to remove other possible contaminants. The virus was precipitated by ultracentrifugation at 155.000g for 2 h at 4°C. The pellets in all tubes were dissolved in 500 µl dH_2O, and all were combined in a single tube to determine the concentration.

Preparation of formulation

To prepare the prototype formulation, a 0.5% gelatin solution was first prepared with deionized water. The solution was stirred with a magnetic stirrer in a 35°C heating block until it appeared colorless and transparent. After adjusting the pH to 7, the solution was filtered through a 0.22 µm membrane and vortexed (Luo et al., 2021; Kadji et al., 2022). The formulation was prepared by adding the crude virus suspension purified in the previous step and the additives. The additives used and their intended uses are listed in Table 1. The prepared formulation was homogenized in a magnetic stirrer and transferred to a glass bottle.

Table 1. Ingredients of the formulation prepared against *Tenebrio molitor* larvae

Ingredients	Purpose of use	Amount (%)
Pure densovirus (TmDNV-TR)	Virulence	70
Beef gelatin	Thermostability	0.5
Tween-80	Surfactant	2
Glycerine	Feeding stimulant	6
Sorbitol	Sweetener	1
dH_2O	Volume complement	20.5

Dose-response test of formulation

The formulation was prepared in five different doses and sprayed on cracked wheat. Contaminated cracked wheat was fed to 10^{th} instar larvae starved for 16 h. Thirty larvae were used for each dose and control group (only dH_2O), and experiments were performed with 3 replicates. Biotest conditions were set as in the crude virus dose-response. Mortality was observed and recorded daily. The crude virus bioassay was completed on day 12, while the formulation bioassay was finished by day 14.

Stability of formulation

The prepared formulation was divided into two and stored at 4°C (refrigerator temperature) and 25°C (storage/room temperature) until the stability tests were performed in glass containers. After 3 and 6 months, it was fed to insects (under the conditions specified in the biotests) and compared with the data from the freshly prepared formulation (given in the previous step) to determine whether it had retained its stability. The treatments were replicated three times.

Data analysis

After mortality rates were calculated using the Abbott formula (Abbott, 1925), lethal dose and time values were determined with probit analysis using SPSS 31.0 software (p<0.05). Median Survival times (ST50) and corresponding confidence intervals were calculated using the log-rank test by Kaplan–Meier analysis.

Results

Dose-response test of crude virus

As a result of the crude virus dose-response, the larvae showed densovirus symptoms (bodies turned black) (Figure 1). In the laboratory, the five doses of crude TmDNV were pathogenic to *T. molitor* larvae, as indicated by the Kaplan–Meier survival curves, which were low and minimally different from each other (X^2= 33.37, log-rank, df=5, p<0.001) (Figure 2). Statistical data analysis revealed an LD_{50}: 1.091 x 10^5 copies/mL^{-1}, LT_{50}: 5 day, LD_{95}: 8.94 x 10^7 copies/mL^{-1}, and LT_{95}: 7 day at the end of the 8th day. (Table 2).

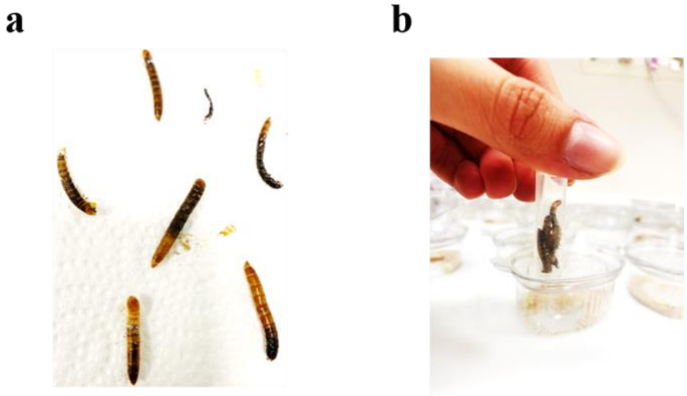

Figure 1. *Tenebrio molitor* larvae died infected with densovirus (TmDNV-TR): a) Larvae bodies were turned black and dead; b) Transfer of dead larvae to microcentrifuge tubes.

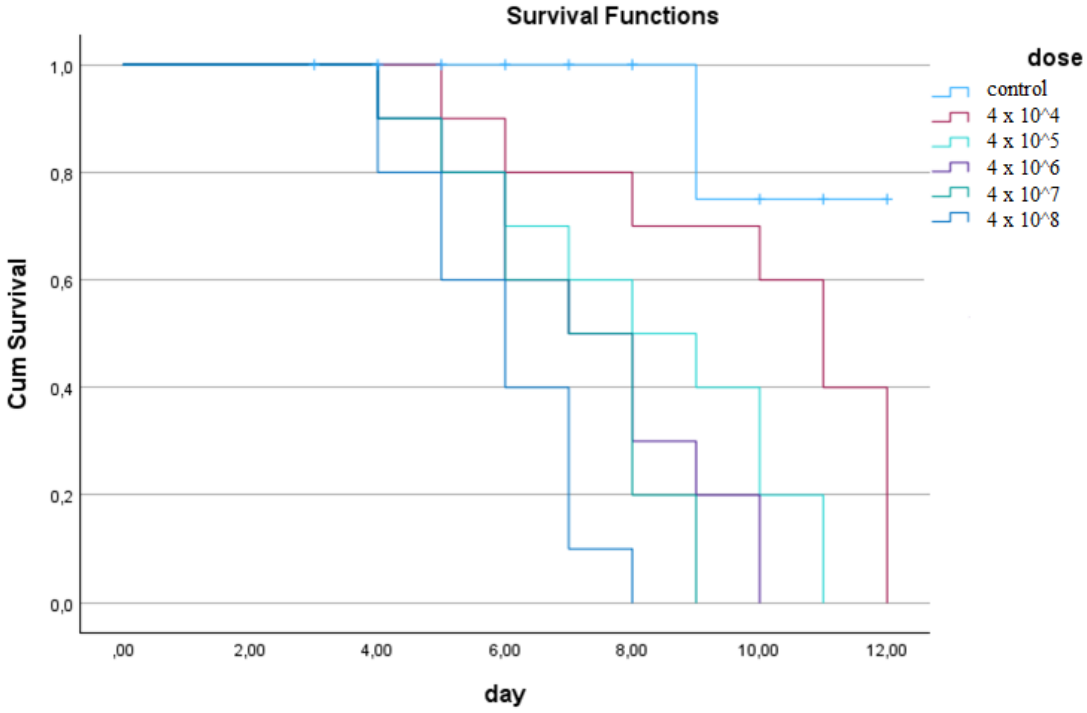

Figure 2. Kaplan-Meier survival curve for 10th instar *Tenebrio molitor* larvae exposed to five different doses (4 × 10^4 to 4 × 10^8 copies/mL^{-1}) of the crude densovirus (TmDNV-TR)

Table 2. Dose-mortality estimated by probit analysis for infectivity of TmDNV-TR isolate on *Tenebrio molitor* larvae

Virus isolate	LD_{50} (copies/mL^{-1})	LT_{50} (day)	LD_{95} (copies/mL^{-1})	LT_{95} (day)	Slope ± SE	*p* value
TmDNV-TR	1.091 x 10^5	5 day	8.94 x 10^7	7 day	0.565±0.055	< 0.05

Dose-response test of formulation

In the laboratory, the five doses of formulation were pathogenic to *T. molitor* larvae, as indicated by the Kaplan–Meier survival curves, which were low and minimally different from each other (X^2= 22.038, log-rank, df=5, p<0.001) (Figure 3). The LD_{50}, LT_{50}, LD_{95}, LT_{95}, slope, and *p* values are shown in Table 3 at the end of the 9th day.

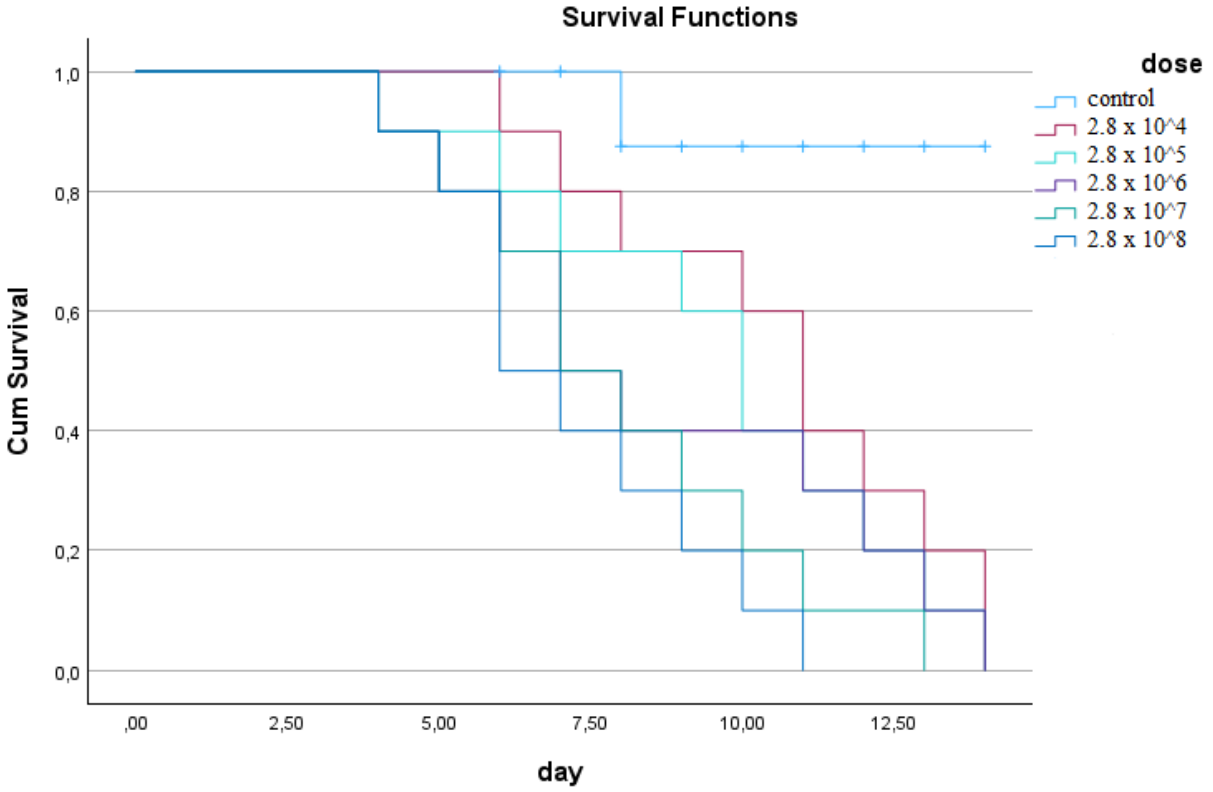

Figure 3. Kaplan-Meier survival curve for 10th instar *Tenebrio molitor* larvae exposed to five different doses (2.8 × 10^4 to 2.8 × 10^8 copies/mL^{-1}) of the formulation

Table 3. Dose-mortality estimated by probit analysis for infectivity of formulation on *Tenebrio molitor* larvae

Formulation	LD_{50} (copies/mL^{-1})	LT_{50} (day)	LD_{95} (copies/mL^{-1})	LT_{95} (day)	Slope ± SE	P value
Tenebriokiller	1.14 x10^5	6 day	1.33 x10^8	9 day	0.536±0.053	< 0.05

Stability of formulation

As given in the previous step, the mortality rate of the fresh formulation (0 months) at the end of the 14th day was 100%, while at 4°C the mortality rate at the end of 3 and 6 months was 100%, and at 25°C the mortality rate at the end of 3 and 6 months was 98% and 95%, respectively (Figure 4).

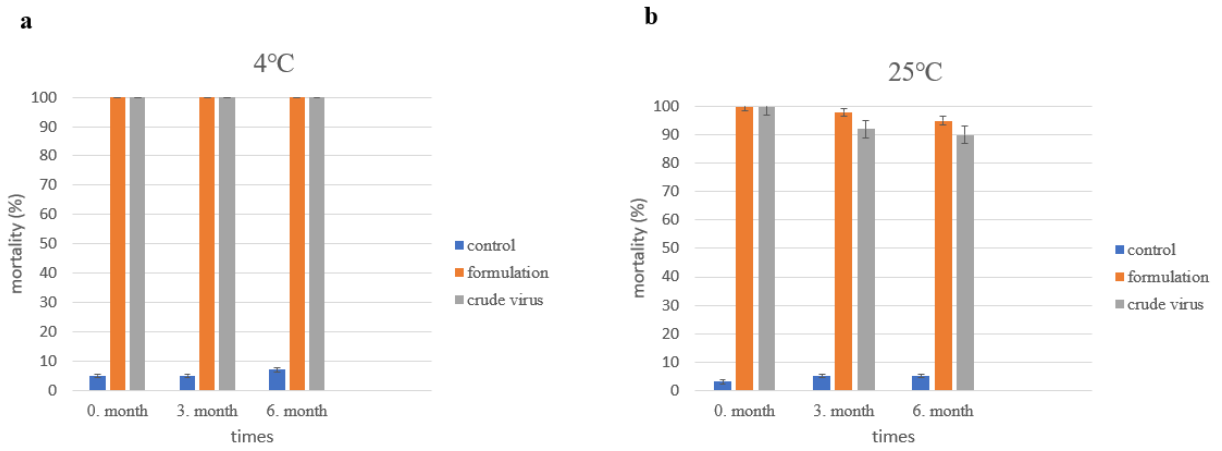

Figure 4. Stability assay of the formulation on 10th instar *Tenebrio molitor* larvae: a) 4°C; b) 25°C (Bars indicate standard error).

Discussion

Two DNA viruses, namely densovirus and iridovirus (Huger, 1969; Kelly et al., 1979; Black et al., 1981; Armien et al., 2023; Aş et al., 2025), and five RNA viruses were identified in *T. molitor* (Hernandez-Pelegrín et al., 2025). The effects of RNA viruses on insect mortality have not been evaluated in the literature. However, analyses of the mortality effects of the two DNA viruses have revealed important differences. The first of these differences is that Densoviruses are known to have rapid oral infectivity (Kittayapong et al., 2001; Rwegoshora & Kittayapong, 2004) but the oral infectivity of iridoviruses is quite low. For this reason, iridoviruses are administered to insects by injection (Williams et al., 2005; Gencer et al., 2020). Iridoviruses can be recombined to increase their oral infectivity by adding a scorpion venom gene (Ozgen et al., 2014; Nalcacıoglu et al., 2016), but as the use of recombinant products as biopesticides is prohibited in many countries, these studies remain at the laboratory scale as pure science. Another difference between iridoviruses and densoviruses is that densoviruses are non-enveloped whereas iridoviruses are enveloped. The higher thermostability of non-enveloped viruses compared to enveloped viruses is important for the shelf life and storage of formulated viruses (Kadji et al., 2022). Densoviruses have been reported to be actively infectious for more than 18 months after the death of their host insects (Tokarev et al., 2020). Densoviruses could therefore be considered an ideal biological control agent. Another difference is the ability to produce viruses *in vitro*. Since the genome of densoviruses (4-6 kb) (Cotmore et al., 2019) is quite small compared to the genome of iridoviruses (212 kb) (Jakob et al., 2001), they can be produced very quickly and in large quantities *in vitro* using a bacterial vector (Guo et al., 2000; Wang et al., 2007). However, in vitro production of iridoviruses depends on insect cell culture (Gencer et al., 2020), which significantly increases the cost of large-scale production.

Huger (1969) was the first to describe a virus infecting *T. molitor*. The viral structures visualised by electron microscopy were thought to be densoviruses. However, no molecular analysis or bioassays were performed. Kelly et al. (1979) and Black et al. (1981) reported that *T. molitor* was infected with iridovirus (type 29) by electron microscopy. They reported that virus replication occurred in the cytoplasm. La Fauce et al. (2008) exposed *Tenebrio molitor* larvae to *Penaeus merguiensis* densovirus isolate and declared that virus replication occurred in 20% of the larvae. Szelei et al. (2011) detected that densovirus spread horizontally between *Acheta domesticus* (L., 1758) (Orthoptera: Gryllidae) and *T. molitor*. During the examination of *T. molitor* larvae, some cytopathic effects were reported, especially in the fat tissue. Gencer et al. (2020) injected both wild-type and recombinant iridovirus (type 6), originally isolated from another insect, into *T. molitor* larvae. Following infection, the larvae exhibited paralysis, darkening of the body, and mortality within three days. Armien et al. (2023) detected a densovirus isolate in *T. molitor* larvae and

identified it by transmission electron microscopy (TEM) and molecular analyses. To maintain the thermostability of crude viruses at different temperatures over time, they need to be formulated (Eroğlu & Demirbağ, 2022). Gelatine is an important stabilizer for the long-term thermal stability of viruses (Kadji et al., 2022). It is a preferred stabilizer in virus formulation or vaccine production due to its high biocompatibility and biodegradability (Su and Wang, 2015). It also offers some advantages, such as being easy to find, cheap, food-compatible, and considered safe to feed (Topuz & Boran, 2018). For this reason, bovine gelatin, which is found in all types of edible food, was used in the formulation of the TmDNV-TR isolate. The reason for adding 0.5% bovine gelatin to the formulation to maintain stability is that this amount is optimal in other virus studies in the literature (Kadji et al., 2022). This is because adding more gelatins will cause the liquid formulation to solidify. Surfactants are substances that reduce surface tension. They are needed to help the formulation spread over the area to which it is applied. In this formulation, 2% Tween-80 was the preferred surfactant. In the literature, up to 5% Tween-80 is usually added to viral formulations (Burges & Jones, 1998; Bayramoğlu et al. 2023). Glycerol is usually used in viral formulations as a feeding stimulant. In the current literature, it has been observed that the amount of glycerol added to viral formulations is between 2% and 10% (Eroğlu & Demirbağ, 2022; Bayramoğlu et al., 2023). Therefore, in this study, a value between those reported in the literature was preferred, and 6% glycerol was added. 1% sorbitol was used as a sweetener to make the formulation attractive to insects. Similarly, the same amount of sorbitol was added to viral formulations in the literature (Bayramoğlu et al., 2023).

The insecticide we have prepared has a spectrum of activity that will be very effective in the industrial sector. This is because pasta, barley, and wheat stored in warehouses are severely damaged by *T. molitor*. As this insect damages the outer packaging of the products and leaves residues in the contents of the product, there is a large loss of product. This loss causes us financial, labor, and crude material losses. In addition, the sale of products contaminated by this insect has many negative effects on human health (Stoops et al., 2016). Indeed, many recent studies have shown that *T. molitor* larvae contain a variety of human-pathogenic bacteria (*Enterobacter* spp., *Klebsiella* spp., *Erwinia* spp., *Pantoea* spp., *Enterococcus* spp., *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., and *Clostridium* spp. (Stoops et al., 2016; Garofalo et al., 2019; Pöllinger-Zierler et al., 2023). This leads to the deterioration of the hygiene of stored products and endangers the health of those who consume them. If this product, which we are currently developing as a prototype, is produced on a large scale, this insect is controlled in the warehouses sprayed with this insecticide, and the aforementioned losses are prevented, many losses in industrial production factories will be prevented, and an important step will be taken in terms of the economy and health in our country.

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