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*Orijinal araştırma (Original article)*

# Tolerances of hybridized entomopathogenic nematode *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) strains to heat and desiccation[1]

## Hibrit entomopatojen nematod *Heterorhabditis bacteriophora* ırklarının yüksek sıcaklık ve su kaybına olan toleransları

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## Summary

Heat and desiccation are two major problems of entomopathogenic nematodes (EPNs) for outdoor applications. The factors decrease their survival and effectivity in the field. Thus, the success ratio drops dramatically especially in hot and drought prone regions. Hybridization of EPNs is a way to overcome these major stress problems. In the present study, heat and desiccation tolerances of ten hybrid strains, which were hybridized from six domestic strains isolated from different climatic regions in Turkey, were determined. The heat tolerance experiments were performed at 32, 34, 36, 38, 40 and 42 °C and desiccation tolerance experiments were performed at PolyEthylene Glycol (PEG) concentrations of 10, 20, 30, 40, 50, 60, 70 and 80 %. The results of the tolerance experiments are shown as mean temperature tolerated by 50% of the population ($MT_{50}$) and mean temperature tolerated by 10% ($MT_{10}$) of the strains for heat testing, and as Lethal Concentration ($LC_{50}$ and $LC_{90}$) for the desiccation test. For the heat tolerance experiments, the results showed that hybrid strains had slightly higher heat tolerance levels than their parents. In the desiccation experiments, it was determined that most of the hybrid strains had greater tolerances than their parents. The outcome of the study was promising for conducting further research and trials.

**Key words:** Heat, desiccation, hybrid, tolerance, *Heterorhabditis bacteriophora*

## Özet

Yüksek sıcaklık ve su kaybı, entomopatojen nematodların (EPN) açık alan uygulamalarındaki en büyük iki olumsuz faktördür. Bu faktörler, arazideki etkinliği ve hayatta kalma oranını düşürmekte ve bu nedenle özellikle sıcak bölgelerde zararlılara karşı başarı oranı ciddi oranda azalmaktadır. EPN'lerin hibridizasyonu, bu iki stres faktörünün önüne geçilmesinde önemli bir yöntemdir. Bu çalışmada, Türkiye'nin değişik coğrafik bölgelerinden izole edilen 6 farklı yerli ırktan hibritlenmiş 10 farklı hibrid ırkın yüksek sıcaklık ve su kaybına olan toleransları belirlenmiştir. Yüksek sıcaklık denemeleri 32, 34, 36, 38, 40 ve 42 ºC'lerde gerçekleştirilirken, su kaybı denemeleri PolyEthylene Glycol (PEG) adlı desikatörün % 10, 20, 30, 40, 50, 60, 70 ve 80 konsantrasyonlarında gerçekleştirilmiştir. Denemelerin sonuçları yüksek sıcaklık için popülasyonun % 50'sinin hayatta kalabildiği ortalama sıcaklık ($MT_{50}$) ve % 10'unun hayatta kalabildiği ortalama sıcaklık ($MT_{10}$) olarak belirtilmişken, su kaybı için lethal konsantrasyon ($LC_{50}$ ve $LC_{90}$) olarak belirtilmiştir. Yüksek sıcaklık denemesinde hibrit ırkların sıcaklığa ebeveynlerinin biraz üzerinde tolerans gösterebildiği saptanırken, su kaybı denemesinde hibrit ırkların hemen hepsinin su kaybına ebeveynlerinden daha iyi tolerans gösterdiği belirlenmiştir. Bu çalışmada elde edilen sonuçlar, bu konuda yapılacak gelecek çalışmalar için ümit verici olarak görülmektedir.

**Anahtar sözcükler:** Yüksek sıcaklık, su kaybı, hibrit, tolerans, *Heterorhabditis bacteriophora*

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## Introduction

For several decades, excessive usage of pesticides and related wrong agricultural applications caused hazardous effects on environment and human heath, which created a focus on using alternative control methods on different agricultural pests. Biological control that has high success rate covers a large proportion within these alternative control methods. Entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae, Steinernematidae) are one of the best known biocontrol agents against especially soil-dwelling insect pests (Gaugler & Kaya, 1990; Georgis, 1990; Ehlers, 1996). Because of their large host spectrum (Peters, 1996), host seeking ability (Lewis et al., 2002; Grewal et al., 1994), application advantages (Georgis, 1990; Koppenhöfer, 2000; Wright et al., 2005) and being environmentally safe (Ehlers, 2001; 2003), EPNs have being used against economically important species, especially in several countries (Ehlers, 1996).

Nevertheless, heat and desiccation are two major stress factors that reduce the effectivity and survival of EPNs and inhibit the widespread usage in outdoor applications (Strauch et al., 2000). There is also a negative impact on shelf life, which limits transportation to other countries and therefore, distribution and usage of the EPNs worldwide. Although adverse effects depend on exposure time, over 40 °C is almost deadly for most EPN species (Koppenhöfer, 2000) and thus, their transportations in warm regions is hardly possible. There are some results on hybridization of EPNs and these studies have revealed a high heritability for these stress factors (Glazer et al., 1991; Strauch et al., 2004; Ehlers et al., 2005; Mukuka et al., 2010c; Mukuka et al., 2010b), which has led to the idea of producing strains with a higher resistance to these stress factors through hybridization. In the present study, hybrid strains of *Heterorhabditis bacteriophora* Poinar 1976 (Rhabditida: Heterorhabditidae) were used because it is one of the most common EPN species found in Turkey (Susurluk et al., 2001). The species has automictic reproduction in its first progeny, which makes the traits genetically more stable especially in liquid culture (Kaya & Gaugler, 1993; Johnigk et al. 2002) and the species has more tolerance to heat than *Steinernema* spp. isolated in Turkey (Susurluk, 2008).

In the present study, heat and desiccation tolerance of hybrid *H. bacteriophora* were determined as mean temperature tolerated by 50% of the population ($MT_{50}$) and mean temperature tolerated by 10% ($MT_{10}$), and as $LC_{50}$ and $LC_{90}$, respectively. Furthermore, a comparison of tolerances to both stress factors of hybrid strains and their parents was performed. Thus, the possibility of genetic inheritance of the traits from parent to new hybrid generations was investigated.

## Materials and Methods

### Hybrid *Heterorhabditis bacteriophora* strains

The ten hybrid strains of *Heterorhabditis bacteriophora* used in this study were obtained through hybridization from strains collected from six different climatic regions of Turkey. The origin of the strains is showned in Table 1. Males and females of the six strains were hybridized with the male and female of the most tolerant strain detected by heat and desiccation tolerances, individually (Table 2) (Mukuka et al., 2010b) to determine if the hybrid strains would be more tolerant than their parents. The hybridization was conducted on bacto agar that had been previously inoculated with the symbiotic bacterium *Photorhabdus luminescens* under *in vitro* conditions. Especially, unfertilized females that carried large and transparent unfertilized eggs without shell were collected for the procedure. The hybridization process including egg isolation as carried out *in vitro,* according to Lunau et al. (1993).

Table 1. Origin and habitat of *Heterorhabditis bacteriophora* (H.b.) strains used in the study

| Strains | Geographic Origins | Habitats |
|---|---|---|
| H.b. 10 | Adana | Cotton |
| H.b. HSU | Şanlıurfa | Wheat |
| H.b. 6 | Antalya | Orchards |
| H.b. HIZ | İzmir | Orchards |
| H.b. 17 | Kırklareli | Wheat |
| H.b. 876 | Çanakkale | Forest |

Table 2. Hybridized parent strains of *Heterorhabditis bacteriophora* used in the study

| Parent strains | Hybrid strains |
|---|---|
| H.b. 6 (Antalya) ♀ X H.b. 876 (Çanakkale) ♂ | H.b. A |
| H.b. 6 (Antalya) ♂ X H.b. 876 (Çanakkale) ♀ | H.b. B |
| H.b. 6 (Antalya) ♀ X H.b. 17 (Kırklareli) ♂ | H.b. C |
| H.b. 6 (Antalya) ♂ X H.b. 17 (Kırklareli) ♀ | H.b. D |
| H.b. 6 (Antalya) ♀ X H.b. HIZ (İzmir) ♂ | H.b. E |
| H.b. 6 (Antalya) ♂ X H.b. HIZ (İzmir) ♀ | H.b. F |
| H.b. 6 (Antalya) ♀ X H.b. HSU (Şanlıurfa) ♂ | H.b. G |
| H.b. 6 (Antalya) ♂ X H.b. HSU (Şanlıurfa) ♀ | H.b. H |
| H.b. 6 (Antalya) ♀ X H.b. 10 (Adana) ♂ | H.b. K |
| H.b. 6 (Antalya) ♂ X H.b. 10 (Adana) ♀ | H.b. L |

**Determination of heat tolerance capabilities**

All hybrid strains were exposed to the following six temperatures: 32, 34, 36, 38, 40 and 42 ºC. The experiments were performed in 24-well plates (each well 1.4 cm diameter and 3 $cm^3$ volume). The experiment was replicated five times.

The hybrid strains, which were stored at +4 ºC in culture flasks, were adapted to the room temperature (24 ºC) for 2 hours before the experiments. After the adaptation, every well of the plate was filled with 500 IJs and 500 µl distilled water, which was at the temperature of the current experiment. All plates were sealed with parafilm and all hybrid strains were exposed to the specific temperature for 2 hours (Mukuka et al., 2010d). Subsequently, all strains were adapted to the room temperature for 24 hours and after the adaptation, dead and live IJs of each strain were counted under a stereomicroscope and mortality of the strains were determined for each temperature.

**Determination of desiccation tolerance capabilities**

Desiccation experiments were also performed in 24-well plates as described above. Different concentrations (10, 20, 30, 40, 50, 60, 70 and 80 %) of PolyEthylene Glycol (PEG) ($HOCH_2$-$CH_2$-(O-$CH_2$-$CH_2$)$_{(n-1)}$-OH) were used as the desiccant. This experiment was also replicated five times.

Each PEG concentration was prepared with sterile Ringer's solution (laboratory standard containing NaCl 9 g, KCl 0.42 g, $CaCl_2$ x $2H_2O$ 0.37 g, $NaHCO_3$ 0.2 g and aqua dest 1000 mL). As in the heat tolerance experiments, 500 IJs were put into the wells of the 24-well plates and filled with 500 µl of the specific PEG concentration. All plates were sealed with parafilm and the hybrid strains were exposed to the different PEG concentrations for 24 h at 25 °C. Because of dehydration, the IJs entered dormancy and it became impossible to distinguish dead or live individuals so all hybrid strains were washed with distilled water and soaked for another 24 hours for rehydration. Afterwards, dead and live IJs were counted and results were recorded as Lethal Concentration ($LC_{50}$ and $LC_{90}$) (Strauch et al., 1994; Mukuka et al., 2010c).

### Statistical analyses

$MT_{50}$ and $MT_{10}$ and $LC_{50}$ and $LC_{90}$ values were calculated through Probit analysis by using BioStat® 2010 software. The correlations between the heat and desiccation tolerances were analyzed with Pearson's correlation coefficient at a 5% confidence level with the program of JMP® 7.0.

## Results

### The most tolerant strain

Strain 6 was determined to be the most tolerant strain to heat and desiccation stresses (Table 3). Thus, the strain-H.b. 6 was hybridized with other strains in the following experiments.

Table 3. Values of the strains to heat and desiccation tolerance as $LC_{50}$-$LC_{90}$ (% PEG) and $MT_{50}$-$MT_{10}$ (ºC), respectively

| Strains | $LC_{50}$ | $LC_{90}$ | $MT_{50}$ | $MT_{10}$ |
|---|---|---|---|---|
| H.b. 6 | 49.06 | 69.94 | 40.50 | 44.06 |
| H.b. 876 | 48.99 | 69.53 | 38.60 | 41.94 |
| H.b. 17 | 46.65 | 64.73 | 40.46 | 43.78 |
| H.b. HIZ | 43.21 | 57.82 | 40.00 | 44.00 |
| H.b. HSU | 42.04 | 54.61 | 40.75 | 43.69 |
| H.b. 10 | 43.04 | 55.84 | 39.27 | 42.58 |

### Heat tolerance

The results of the heat tolerance experiments showed that the most tolerant hybrid strain was H.b. A ($MT_{50}$=40,65; $MT_{10}$=47,19), followed by H.b. B ($MT_{50}$=40,57; $MT_{90}$=44,06) and that only these two hybrid strains exceeded the heat tolerance levels of their parents. Moreover, the most susceptible three hybrids strains were H.b. L $MT_{50}$=35,19; $MT_{10}$=41,13), H.b. F ($MT_{50}$=27,46; $MT_{10}$=41,44) and H.b. E ($MT_{50}$=38,05; $MT_{10}$=41,65). With exception of the strains A and B, all hybrid strains were less tolerant to heat stress than the parental strains (Table 4).

Table 4. Heat tolerances of the parent and hybrid strains as $MT_{10}$ and $MT_{50}$ (ºC)

| Parent and Hybrid Strains | $MT_{50}$ | $MT_{10}$ |
|---|---|---|
| H.b. 6 (the best strain) | 40.50 | 44.06 |
| H. b. 876 | 38.60 | 41.94 |
| H.b. A (H.b. 6 ♀ x H.b. 876 ♂) | 40.65 | 47.19 |
| H.b. B (H.b. 6 ♂ x H.b. 876 ♀) | 40.57 | 44.16 |
| H.b. 17 | 40.46 | 43.78 |
| H.b. C (H.b. 6 ♀ x H.b. 17 ♂) | 40.10 | 43.09 |
| H.b. D (H.b. 6 ♂ x H.b. 17 ♀) | 37.00 | 41.17 |
| H.b. HIZ | 40.00 | 44.00 |
| H.b. E (H.b. 6 ♀ x H.b. HIZ ♂) | 38.05 | 41.65 |
| H.b. F (H.b. 6 ♂ x H.b. HIZ ♀) | 37.46 | 41.44 |
| H.b. HSU | 40.75 | 43.69 |
| H.b. G (H.b. 6 ♀ x H.b. HSU ♂) | 38.56 | 43.01 |
| H.b. H (H.b. 6 ♂ x H.b. HSU ♀) | 39.94 | 43.27 |
| H.b. 10 | 39.27 | 42.58 |
| H.b. K (H.b. 6 ♀ x H.b. 10 ♂) | 38.59 | 41.87 |
| H.b. L (H.b. 6 ♂ x H.b. 10 ♀) | 35.19 | 41.13 |

### Desiccation tolerance

In the desiccation tolerance experiment, the most tolerant hybrid strain was H.b. F ($LC_{50}$=54,52; $LC_{90}$=86,74) for both $LC_{50}$ and $LC_{90}$ values. This strain was followed by H.b. C ($LC_{50}$=57,81; $LC_{90}$=75,92)

and H.b. B ($LC_{50}$=55,00; $LC_{90}$=76,56). The most susceptible hybrid strains were H.b. D ($LC_{50}$=42,19; $LC_{90}$=74,28), H.b. L ($LC_{50}$=43,91; $LC_{90}$=77,30) and H.b. A ($LC_{50}$=52,37; $LC_{90}$=72,30). Results for desiccation tolerance showed that all hybrid strains were more tolerant than their parents, except strains D and L. (Table 5).

Table 5. Desiccation tolerances of the parent and hybrid strains as $LC_{10}$ and $LC_{50}$ (% PEG)

| Parent and Hybrid Strains | $LC_{50}$ | $LC_{90}$ |
|---|---|---|
| H.b. 6 (the best strain) | 49.06 | 69.94 |
| H. b. 876 | 48.99 | 69.53 |
| H.b. A (H.b. 6 ♀ x H.b. 876 ♂) | 52.37 | 72.30 |
| H.b. B (H.b. 6 ♂ x H.b. 876 ♀) | 55.00 | 76.56 |
| H.b. 17 | 46.65 | 64.73 |
| H.b. C (H.b. 6 ♀ x H.b. 17 ♂) | 57.81 | 75.92 |
| H.b. D (H.b. 6 ♂ x H.b. 17 ♀) | 42.19 | 74.28 |
| H.b. HIZ | 43.21 | 57.82 |
| H.b. E (H.b. 6 ♀ x H.b. HIZ ♂) | 54.38 | 74.85 |
| H.b. F (H.b. 6 ♂ x H.b. HIZ ♀) | 54.52 | 86.74 |
| H.b. HSU | 42.04 | 54.61 |
| H.b. G (H.b. 6 ♀ x H.b. HSU ♂) | 53.45 | 76.02 |
| H.b. H (H.b. 6 ♂ x H.b. HSU ♀) | 54.36 | 70.85 |
| H.b. 10 | 43.04 | 55.84 |
| H.b. K (H.b. 6 ♀ x H.b. 10 ♂) | 55.66 | 75.83 |
| H.b. L (H.b. 6 ♂ x H.b. 10 ♀) | 43.91 | 77.30 |

**Correlations between heat and desiccation tolerances of the parent and the hybrid strains**

There was a statistically significance correlation between the heat and desiccation tolerances of the hybrid strains (y= 3.5793x-81.46; r=0.76; p<0.0001). Likewise, there was also a significant relationship between the heat and desiccation tolerances of the parent strains (y= 3.8383x-106.02; r=0.75; p<0.0001). Correlation of the hybrid strains was slightly greater than for the parents (Figure 1). Mean tolerated temperatures (ºC) and lethal concentrations (%) (Fig 1) were obtained from the means of $MT_{50}$-$MT_{90}$ and $LC_{50}$-$LC_{90}$, respectively.

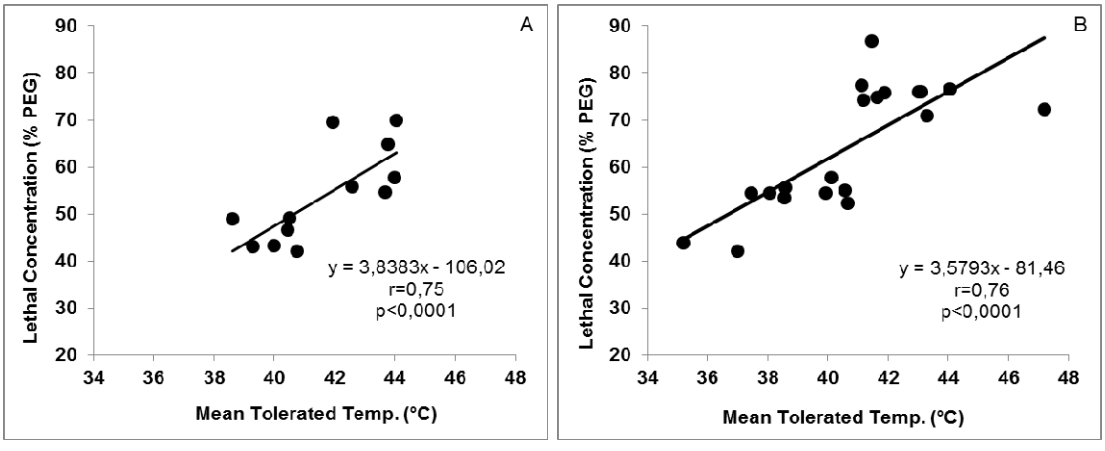


Figure 1. Correlations between heat and desiccation tolerances of the parent (A) and the hybrid strains (B).

## Discussion

Heat and desiccation are the two major stress factors which reduce survival and effectivity of EPNs and lower their shelf life (Strauch et al., 2000; Mukuka et al., 2010). Overcoming their negative effects is extremely important for successful and effective biocontrol in greenhouse and field applications. The main purpose of this study was to produce strains through hybridization that have high tolerance to heat and desiccation.

Among the six strains isolated from different climatic regions, strain H.b. 6 from Antalya showed the most tolerance to the two stress factors.This result was in accordance with the climatic regime of Antalya. Results of the whole study showed that especially for desiccation tolerance, most of the hybrid strains were more resistant to heat and desiccation than their parents without an adaptation phase to these stress factors. Strauch et al. (2000) found that without an adaptation phase to desiccation tolerance, almost all parent strains had higher tolerances to desiccation than hybrid strains. With an adaptation phase, all hybrid strains achieved a higher tolerance. As reported by Strauch et al. (2000), results could be explained by a negative heterosis in non-adapted strains and higher phenotypic variance in adapted strains. Strauch et al. (2000) also stated that for more tolerance, parent strains should be isolated from warm and dry regions such as the Middle East or Turkey, likewise in the present study.

In contrast to the study of Strauch et al. (2000), Mukuka et al. (2010d) documented similar results to those reported in the present study. They demonstrated an almost 3 °C increase in tolerance levels which indicates that hybrid strains showed higher tolerances than their parents. Similar results were also achieved in desiccation tolerance in that tolerances were higher in hybrid strains than in their parents after several hybridizations with more than 40 strains.

In the recent study of Anbesse et al. (2013), although the aim of that study was to stabilize the tolerance, they also managed to improve heat and desiccation tolerance through hybridization. However, desiccation tolerance improved only with selection pressure in both *in vivo* and *in vitro.* Without selection pressure, desiccation tolerance was not different between batches.

Results of the correlations between heat and desiccation tolerances indicated that there was no major difference between the parent (r=0.75) and hybrid strains (r=0.76), which indicates that hybridization of the parents had no effect on the correlation between the stress factors. Such a correlation has not been assessed before, so this is the first report on the possible correlation between heat and desiccation tolerance. However, Mukuka et al. (2010d) reported no correlation between the tolerance with or without adaptation to heat tolerances and no correlation between heat tolerance and mean annual temperature at the place of origin of the strain.

The present study is also the first report for hybridization of EPNs and determining heat and desiccation tolerances of hybrids strains from Turkey. Offering new and effective biocontrol agents will contribute to the use of alternatives to pesticides and promote integrated pest management (IPM) for agriculture in developing countries, and also in Turkey. Although the results of the current study were quite encouraging, more detailed studies need to be performed on stability of tolerance to these two major stress factors and other traits such as host finding, foraging, effectivity and reproduction capability. Moreover, although there have been relatively few studies around the world on this subject (Glazer et al., 1991; Shapiro et al., 1996; Somasekhar et al., 2002; Strauch et al., 2004; Ehlers et al., 2005; Mukuka et al., 2010b; c; d), the results of the present study were very encouraging for the undertaking of further research efforts in Turkey.

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