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Article

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Simple Summary: The Colorado potato beetle is a highly dangerous pest of potatoes and other plants of the Solanaceae family when its main food source is unavailable. Climate change significantly affects the behavior and distribution of this pest, increasing its harmfulness. High fertility, voracity, and ecological plasticity underscore the importance of developing integrated and sustainable pest control strategies in the context of climate change. Insecticides are becoming less effective due to the emergence of resistant populations. A promising alternative to chemical insecticides is entomopathogenic fungi (EPF), which offer a sustainable and environmentally friendly approach to controlling pest populations.

Abstract: This article presents the results of isolating and identifying EPF species and screening thermotolerant and highly virulent EPF strains that are promising for controlling Colorado potato beetle populations. Soil samples were collected from southern Kazakhstan for the isolation of fungi used in the experiments. Using the insect bait method, 41 isolates of EPF were obtained from the soils of southern Kazakhstan. PCR analysis based on ITS and TEF loci (elongation factor Ef1a) showed that all the isolates belong to the species B. bassiana. However, analysis of the ITS locus revealed two cryptic species: *B. bassiana* and *B. pseudobassiana*. No other fungal species were detected, likely due to the insufficient informativeness of the ITS and TEF loci in distinguishing species. Further studies with other loci are required. As a result of selecting natural isolates for virulence and thermotolerance, the following strains were identified: B13, B14, B15, B22, B23, B24, B25, B23-23, Bc4, Bc7, and Bc8. These strains showed high virulence (100% on the 11th day after treatment) and tolerance to suboptimal temperatures (+15°C and +30°C). Therefore, they are considered the most promising for use in the context of climate change. The data suggest that, despite the source of the isolates, EPF have great potential for controlling Colorado potato beetle populations, as the majority of isolates lead to fatal outcomes. Their geographical origin primarily influences their temperature preferences, which negatively affect their effectiveness in field conditions.

Keywords: Colorado potato beetle; climate change; biological plant protection; entomopathogenic fungi; virulence; thermotolerance; *B. bassiana*; B. *pseudobassiana*

1. Introduction

The Colorado potato beetle (*Leptinotarsa decemlineata* Say) was first described in 1824 and initially named the nightshade leaf beetle. It now occupies a special place among the phytophagous insects that damage cultivated plants [1]. It is one of the most striking examples of zoological species that

have thrived in artificial ecosystems, illustrating the biological features of harmful insect phytophages under conditions of anthropogenic agroecosystem transformation [2].

This beetle became widely known primarily due to its "ecological explosion", with an unprecedented territorial expansion in both rate and scale. This began in the mid-19th century when the previously harmless leaf beetle shifted from wild thorny nightshades to cultivated potatoes [3]. It causes significant damage to potatoes and eggplants (*Solanum* species), and, to a lesser extent, tomatoes (*Lycopersicon* species), which are primarily consumed by adults. The beetle is also potentially dangerous to pepper, physalis, melon pear, and tobacco crops [4].

The beetle's preference for potatoes over other nightshade crops results in an increased concentration of pests in potato fields, higher female fertility when feeding on potato leaves, and high larval survival rates during development. As a result, the pest remains a major scourge of potato farming in many countries, including Kazakhstan [5].

In Kazakhstan, the Colorado potato beetle was first detected in 1973 in the Atyrau region, in 1977 in the Aktobe region, in 1978 in the Kostanay region, and in 1989 in the Almaty region. Frequent stormy winds contributed to the widespread dispersal of the pest in the summer. For example, in 1982, a stormy wind from the west spread the Colorado potato beetle across one-quarter of the North Kazakhstan region. In 1986, pest outbreaks were recorded in potato fields in Zhezkazgan and Karaganda, and in 1988-89 in the Almaty, Zhambyl, and Chimkent regions. By the early 1990s, the Colorado potato beetle had spread throughout all regions and had become the most dangerous pest of potato crops. Since then, many areas of the country have seen massive pest population outbreaks, leading to catastrophic potato crop losses and making the issue of protecting potatoes from this pest a matter of national importance [6].

This pest has high economic significance due to its high voracity and fertility [1,7]. Additionally, its extreme ecological plasticity, determined by genetic and physiological polymorphism, allows the Colorado potato beetle to easily adapt to environmental changes and technological impacts, particularly pesticides, while maintaining high potential viability and reproduction rates [8].

The need for phytosanitation is exacerbated by climate change, which significantly alters the pest's behavior and distribution. As a result, scientists have begun to model the possible impacts of climate change on the pest and describe potential consequences: 1) Global warming may allow the Colorado potato beetle to spread to regions where it was previously not found. 2) Rising average temperatures may extend the growing season and increase the number of beetle generations per year, leading to larger population sizes. 3) The beetles may adapt their behavior in response to climate change, such as changing their feeding locations or seeking shade during particularly hot days to avoid heat stress. 4) Variations in rainfall amounts and distribution may affect plant health, making them more susceptible to beetle damage. For example, plants may be less resistant to pest attacks in drought conditions [9–17].

These impacts highlight the importance of developing integrated and sustainable pest control strategies in the context of climate change. Currently, the Colorado potato beetle is primarily controlled using chemical and agrotechnical methods [18]. The list of insecticides approved for use on potatoes for Colorado potato beetle control is quite extensive, but it is well known that chemical methods have a number of significant drawbacks, the most important of which are environmental pollution and the accumulation of initial compounds, metabolites, and degradation products in soil, water, the atmosphere, and living organisms.

Moreover, among harmful insects, the Colorado potato beetle shows the fastest resistance to chemical insecticides, which exacerbates the problem related to anti-resistance protection strategies [19–23]. The negative impact of pesticides on the biosphere and the need to reduce chemical usage in agriculture have led to the search for alternative pest control methods. The most attractive alternative to chemical pesticides is natural agents (entomophages and microorganisms), which are inherently safe for humans and the environment [24–26].

Compared to other biological agents, entomopathogenic fungi (EPF) have the greatest potential as they cause severe epizootics in insects, more so than bacteria and viruses. Additionally, the use of EPF offers several advantages, including the following: 1) fungi are easily cultivated and can be

produced in large quantities at a relatively low cost; 2) they infect insects through direct penetration of the cuticle and can therefore act as contact insecticides; 3) there are no issues related to resistance among insect populations due to the complex pathogenesis process; 4) fungi have the ability to degrade chemical pesticide residues; 5) there is potential for enhancing fungal stability in the environment to ensure long-term effectiveness; 6) fungi can colonize plants and establish symbiotic relationships as endophytes, thereby increasing plant resistance to pests and diseases; 7) a lack of impact on mammals, thus reducing environmental toxicity; and 8) compatibility with insecticides [27–44].

To date, around 1,000 species of EPF are known worldwide, many of which have adapted to a lifestyle reliant on insects [44–46]. They belong to various taxonomic groups such as *Oomycetes, Microsporidia, Chytridiomycota, Entomophthoromycota, Basidiomycota*, and *Ascomycota* [46]. Among these, anamorphic ascomycetes such as *Beauveria, Metarhizium, Lecanicillium*, and *Isaria*, which infect a wide range of insects and are widely distributed, have significant potential in applied work [48–50].

EPF have been used to control economically important pests for nearly 200 years, and today, the number of mycoinsecticides based on them exceeds 100 [49–52]. However, the total share of biopesticides on the global pesticide market does not exceed 6%, which is significantly lower than the share of chemical insecticides [53]. To expand the range of mycoinsecticides and mycoacaricides, more work is being carried out to search for supervirulent strains with a selective spectrum of action [54–59]. Special attention is paid to the ability of strains to retain their pathogenic properties under unfavorable environmental conditions [60–65].

Due to the high sensitivity of EPF to environmental conditions, their use in the field may not yield the desired effect. Their effectiveness can be improved by using biopolymers (polysaccharides, polypeptides, and proteins), which can extend the viability of conidia in the field [66,67]. Cellulose, chitosan, alginate, and starch are often used for this purpose [68–71].

In this regard, the goal of this study is to select thermotolerant and highly virulent strains of EPF that are promising for controlling Colorado potato beetle populations, as well as finding methods to preserve their viability in the sharply continental and arid climate of Kazakhstan to expand the adaptability of agriculture to climate change.

2. Materials and Methods

2.1. Sample Collection and Fungal Isolation

EPF can be isolated from insect cadavers, plants, and soil [49,52,72–75]. Since soil is the natural habitat for most species of entomopathogenic fungi, soil samples were collected from various locations in southern Kazakhstan in 2023-2024 using a soil probe to a depth of 20 cm and then placed in sterile ziplock bags. The collected soil was mechanically ground and sieved to remove foreign materials. It was then stored in a refrigerator at 4°C for further analysis.

There are two methods for isolating entomopathogenic fungi: selective media and insect baits [76,77]. The most effective method involves using susceptible larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) and *Tenebrio molitor* (Coleoptera: Tenebioidae) [78–83]. Larvae of the large mealworm beetle Tenebrio molitor were used to isolate entomopathogenic fungi. From each collected soil sample, 250 grams were placed in a plastic container (with 5 replicates). Five Tenebrio molitor larvae were also added along with food to prevent premature death without infection. The containers were stored at 25°C. Every 3-4 days, the larvae were monitored for signs of mycosis to confirm infection. Infected larvae were placed in a moist chamber (at 25°C for 7 days) to promote the appearance of mycosis. When signs of mycosis appeared, conidia were collected from the surface of the larvae and transferred to Sabouraud dextrose agar for further growth. Re-seeding was performed as needed until a pure culture was obtained.

2.2. EPF Species Identification

EPF species identification was carried out using classical light microscopy and molecular genetic methods. The isolates were cultured on Sabouraud dextrose agar for 14 days [84]. Highly informative molecular markers were used in a multilocus approach to identify species of *Beauveria*, such as translation elongation factor-1α (TEF-1α) and internal transcribed spacer (ITS) [85–88]. The results were compared with deposited cultures in GenBank using the BLAST method via the NCBI server.

2.3. Determining the Effect of Temperature on the Radial Growth of Anamorphic Ascomycetes

To determine the effect of temperature on the radial growth intensity of fungi in the surface culture, Petri dishes with Sabouraud dextrose agar were prepared 48 hours in advance [89]. The fungi were inoculated by pricking the center of the Petri dish with an even layer of medium. The dishes were placed in an incubator at fixed temperatures (+10°C, +15°C, +20°C, +25°C, +30°C, and +35°C). For 29 days, colony measurements were taken every 2 days in two perpendicular directions (crosswise) to determine colony diameter. The experiments were repeated four times.

2.4. Determining the Viability of Conidia

The germination rate of conidia of the studied fungal strains was determined in a water droplet. Two drops of conidial suspension of the tested strain were placed on a coverslip and placed in a moist chamber (Petri dish with a damp filter) in an incubator at +20°C and +25°C. The slides were observed every 12 hours under a microscope in five fields of view for 72 hours. For greater accuracy, conidia were counted in multiple drops of the suspension, with four replicates.

2.5. Preparation of the Working Suspension and Determination of Titer Using the Goryaev Chamber

To prepare the working suspension, dry fungal biomass was scraped from the Sabouraud dextrose agar medium using a scalpel. The biomass was placed in a glass test tube, covered with 2 ml of cold sterile water, and thoroughly mixed. From this mixture, 1 ml was taken using a pipette and added to a prepared sterile test tube containing 9 ml of cold sterile water. The resulting suspension was thoroughly mixed until it became cloudy. A drop of the semi-preparative form was applied to the Goryaev chamber, and excess liquid was removed with filter paper. The spore titer in 1 ml of suspension was determined by counting the spores in large squares using the following formula:

$$T = \frac{\sum \text{spore } X \, 50}{k} \, X \, 5000$$

T—spore titer in spores/ml;
$\sum$ spore —the sum of spores counted in large squares;
k—the number of large squares with counted spores.

If necessary, the suspension was diluted with water to the required concentration or a new batch was prepared. The prepared solution was used within 1-2 hours, as further germination of fungal conidia reduces the effectiveness of the treatment [90].

2.6. Laboratory Evaluation of Biological Activity

To determine the biological activity of EPF against Colorado potato beetle larvae, test insects without mechanical or other visible damage were placed in cages at 5 individuals per cage. Each cage was a 1000 ml plastic container covered with milling gauze. The insects were infected at room temperature. The experiments were repeated four times.

First, a control group was treated with distilled water to create identical conditions for all insects, and then the test insects were treated with a fungal suspension. A conidial titer of 1×10^7 was used in the preliminary screening tests. The containers were inspected daily for 20 days after treatment, and dead individuals were collected. The cadavers were then placed on slides in a moist chamber to determine the cause of death and the level of fungal colonization.

Biological effectiveness was calculated using Abbott's formula [91]:

$$C = \frac{100(A-B)}{A}$$

C—percentage of pest mortality;
A—average number of individuals before treatment;
B—average number of individuals after treatment.

2.7. Statistical Analysis

Statistical analysis was performed using the analysis of variance method using Microsoft Office Excel spreadsheets and the IBM statistical application package.

3. Results

3.1. Species Composition of Entomopathogenic Fungi

To isolate entomopathogenic micromycetes, more than 150 soil samples were collected from various points of agrocenoses in the region. In laboratory conditions, larvae of the yellow mealworm (*Tenebrio molitor* L.) were placed in these soil samples as bait for EPF (Figure 1). After 29 days, 647 out of 865 larvae died. Signs of mycosis appeared in more than 60 larvae from different soil samples out of the 647 dead larvae. In total, 41 isolates of EPF were obtained in pure culture.

An analysis of the species composition of the isolated cultures based on morphological characteristics showed that most of the isolates belonged to the genus *Beauveria*. During the isolation of EPF into pure culture, the cultural and morphological features of new isolates were also examined.

Figure 1. Soil samples with mealworm larvae (*Tenebrio molitor* L).

It was established that all newly isolated strains grow well on Sabouraud nutrient medium. Most of the isolates exhibited morphology characteristic of the genus *Beauveria*, specifically round colonies of white or beige color with a darker center. The mycelium is fluffy or felt-like and white in color (Figure 2).

Figure 2. Morphology of colonies of entomopathogenic micromycetes isolated from soils of agrocenoses of the Turkestan region on Sabouraud nutrient medium.

The size of conidia from the isolated strains was assessed to identify cryptic species. However, no significant differences in spore size were found. Therefore, PCR analysis was conducted on the ITS and TEF loci to clarify the taxonomic status of natural isolates. PCR analysis of the nuclear DNA locus tef (elongation factor EF1α) showed that all isolated strains belong to the species *B. bassiana* (Table 1). However, analysis of the ITS locus revealed two cryptic species: *B. bassiana* and *B. pseudobassiana*. The overwhelming majority of the isolates belong to *B. bassiana*, 85% (35 isolates), with B. pseudobassiana being the second most common at 15% (6 isolates). No other fungal species were detected. This is likely because their distribution is limited by specific environmental conditions or hosts.

Table 1. Species composition of natural isolates of EPF isolated from the soils of southern Kazakhstan.

№	Isolates	Type species in Genbank by ITS locus	Number of genes read	Other similar species	Type species in Genbank for TEF locus	Number of genes read	Other similar species
1	B9	*B. pseudobassiana* ARSEF 3405	614	*B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1251	*B. bassiana* isolate ARSEF 815 *Beauveria* sp. KVL_03_136
2	B10	*B. pseudobassiana* ARSEF 3405	940	*B. majiangensis* GZAC GZU1214 *B. kipukae* ARSEF 7032	*B. bassiana* ARSEF 2860	1250	*B. bassiana* isolate ARSEF 815 *Beauveria* sp. KVL_03_136
3	B11	*B. pseudobassiana* ARSEF 3405	657	*B.majiangensis* GZAC GZU1214 *B. bassiana* ARSEF 1564	*B. bassiana* ARSEF 2860	1244	*B. bassiana* isolate ARSEF 815 *Beauveria* sp. KVL_03_136

				B.australis ARSEF 4598			
4	B12	*B. bassiana* ARSEF 1564	352	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B.majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1016	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
5	B13	*B. bassiana* ARSEF 1564	588	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B.majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1018	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
6	B14	*B. bassiana* ARSEF 1564	701	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. pseudobassiana* ARSEF 3405 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1314	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
7	B15	*B. bassiana* ARSEF 1564	465	*B.australis* ARSEF 4598 *B.varroae* ARSEF 8257 *B.majiangensis* GZAC GZU1214 *B.amorpha* ARSEF 2641	*B. bassiana* ARSEF 2860	1029	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1

				B. lii ARSEF 11741			
8	B16	*B. bassiana* ARSEF 1564	491	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1028	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
9	B17	*B. bassiana* ARSEF 1564	636	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741 *B.amorpha* ARSEF 2641 *B. malawiensis* IMI 228343	*B. bassiana* ARSEF 2860	1020	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
10	B18	*B. bassiana* ARSEF 1564	690	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1014	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
11	B19	*B. bassiana* ARSEF 1564	343	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257	*B. bassiana* ARSEF 2860	1017	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5

				B. majiangensis GZAC GZU1214			*B. bassiana* isolate BbDm-1
12	B20	*B. bassiana* ARSEF 1564	314	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1025	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
13	B21	*B. bassiana* ARSEF 1564	646	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741 *B. malawiensis* IMI 228343 *B.amorpha* ARSEF 2641	*B. bassiana* ARSEF 2860	1015	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
14	B22	*B. bassiana* ARSEF 1564	319	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1020	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
15	B23	*B. bassiana* ARSEF 1564	462	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis*	*B. bassiana* ARSEF 2860	1016	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5

				GZAC GZU1214			*B. bassiana* isolate BbDm-1
16	B24	*B. bassiana* ARSEF 1564	646	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *Beauveria majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741 *B. malawiensis* IMI 228343 *B.amorpha* ARSEF 2641	*B. bassiana* ARSEF 2860	1028	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
17	B25	*B. bassiana* ARSEF 1564	437	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1019	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
18	B26	*B. bassiana* ARSEF 1564	524	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1023	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
19	B28	*B. bassiana* ARSEF 1564	384	*B. australis* ARSEF 4598 *B. majiangensis* GZAC GZU1214 *B. varroae* ARSEF 8257	*B. bassiana* ARSEF 2860	1017	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1

20	B29	*B. bassiana* ARSEF 1564	575	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B.amorpha* ARSEF 2641 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1016	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
21	B30	*B. bassiana* ARSEF 1564	425	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1028	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
22	B53	*B. bassiana* ARSEF 1564	352	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1011	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
23	B23-1	*B. bassiana* ARSEF 1564	472	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1019	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
24	B23-2	*B. bassiana* ARSEF 1564	296	*B. australis* ARSEF 4598 *B. varroae*	*B. bassiana* ARSEF 2860	1020	*B. bassiana* isolate ARSEF 815

				ARSEF 8257 B. majiangensis GZAC GZU1214 B. pseudobassiana ARSEF 3405			Beauveria sp. KVL_03_136
25	B23-3	B. pseudobassiana ARSEF 3405	724	B. majiangensis GZAC GZU1214	B. bassiana ARSEF 2860	1012	B. bassiana isolate ARSEF 815 Beauveria sp. KVL_03_136
26	B23-9	B. pseudobassiana ARSEF 3405	574	B. bassiana ARSEF 1564 B. majiangensis GZAC GZU1214 B. australis ARSEF 4598 B. varroae ARSEF 8257 B.a malawiensis IMI 228343 B. kipukae ARSEF 7032 B. brongniartii ARSEF 617	B. bassiana ARSEF 2860	1021	B. bassiana isolate ARSEF 815 Beauveria sp. KVL_03_136
27	B23-11	B. bassiana ARSEF 1564	266	B. pseudobassiana ARSEF 3405 B. australis ARSEF 4598 B. majiangensis GZAC GZU1214	B. bassiana ARSEF 2860	1015	B. bassiana isolate ARSEF 815 B. bassiana isolate Bb5 B. bassiana isolate BbDm-1

				B. varroae ARSEF 8257			
28	B23-13	*B. bassiana* ARSEF 1564	394	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1013	*B. bassiana* isolate ARSEF 815 *Beauveria* sp. KVL_03_136
29	B23-14	*B. bassiana* ARSEF 1564	640	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1022	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
30	B23-17	*B. bassiana* ARSEF 1564	755	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. malawiensis* IMI 228343 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1029	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
31	B23-18	*B. bassiana* ARSEF 1564	846	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis*	*B. bassiana* ARSEF 2860	1022	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5

				GZAC GZU1214 *B.amorpha* ARSEF 2641 *B. lii* ARSEF 11741			*B. bassiana* isolate BbDm-1
32	B23-23	*B. bassiana* ARSEF 1564	846	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B.amorpha* ARSEF 2641 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1016	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
33	B23-25	*B. pseudobassiana* ARSEF 3405	882	*B. majiangensis* GZAC GZU1214 *B. kipukae* ARSEF 7032	*B. bassiana* ARSEF 2860	1013	*B. bassiana* isolate ARSEF 815 *Beauveria* sp. KVL_03_136
34	B23-11	*B. bassiana* ARSEF 1564	825	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B.amorpha* ARSEF 2641 *B. malawiensis* IMI 228343 *B. lii* ARSEF 11741 *B. caledonica* BCRC 32867	*B. bassiana* ARSEF 2860	1017	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1

35	Bc2	*B. bassiana* ARSEF 1564	432	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B.amorpha* ARSEF 2641	*B. bassiana* ARSEF 2860	1018	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
36	Bc3	*B. bassiana* ARSEF 1564	394	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741	*B. bassiana* ARSEF 2860	1019	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
37	Bc4	*B. bassiana* ARSEF 1564	652	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B. lii* ARSEF 11741 *B.amorpha* ARSEF 2641	*B. bassiana* ARSEF 2860	1024	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
38	Bc5	*B. bassiana* ARSEF 1564	343	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1014	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1

39	Bc6	*B. bassiana* ARSEF 1564	329	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1020	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
40	Bc7	*B. bassiana* ARSEF 1564	626	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214 *B.amorpha* ARSEF 2641 *B. malawiensis* IMI 228343	*B. bassiana* ARSEF 2860	1022	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1
41	Bc8	*B. bassiana* ARSEF 1564	386	*B. australis* ARSEF 4598 *B. varroae* ARSEF 8257 *B. majiangensis* GZAC GZU1214	*B. bassiana* ARSEF 2860	1026	*B. bassiana* isolate ARSEF 815 *B. bassiana* isolate Bb5 *B. bassiana* isolate BbDm-1

3.2. Influence of Air Temperature on the Growth AND Viability of EPF

An important aspect of selecting promising producer strains is determining their temperature preferences. The resistance of fungal cultures to suboptimal temperatures significantly affects the efficacy of mycoinsecticides. Depending on the timing and location of application, strains with varying degrees of thermotolerance are required.

To determine the effect of temperature on the radial growth of fungi in surface culture, temperatures in the range of +10°C, +15°C, +20°C, +25°C, +30°C, and +35°C were used.

At +10°C, the best growth was shown by isolates B10, B11, B12, B24, B25, and B26. In the overall sample of cultures, almost all isolates exhibited growth at this temperature. At +15°C, the same trend was observed as at the previous temperature, but the colony diameter was significantly larger than at +10°C (Figure 3). At +20°C, on the 29th day after sowing, the maximum colony growth was observed in isolates B18, B19, B20, and Bc6. The colony diameter at this temperature ranged from 44.1 mm to 69.6 mm. For this sample of isolates, the maximum colony growth was observed at +25°C. By the end of the cultivation period—on the 29th day—the best results were shown by isolates B9, B10, B14, B18, B19, B20, B25, B29, B30, B23-13, B23-14, B23-23, Bc4, Bc5, and Bc6. The diameter of these colonies exceeded 80 mm. At +30°C, the intensity of radial growth decreased compared to +25°C. As

a result, at +30°C, the maximum colony growth was shown by isolates B13, B14, B15, B16, B18, B19, B20, B22, B23, B24, B25, B26, B53, B23-2, B23-3, B23-11, B23-13, B23-14, B23-23, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7, and Bc8. Therefore, in nature, even at a constant temperature of 30°C for a month, the fungus is capable of not only maintaining viability but also growing quite vigorously. At +35°C, the lowest growth intensity was observed. Analysis of the obtained data showed that the isolates with increased tolerance to this temperature include B13, B14, B15, B22, B23, B24, B25, B23-23, Bc4, Bc7, and Bc8.
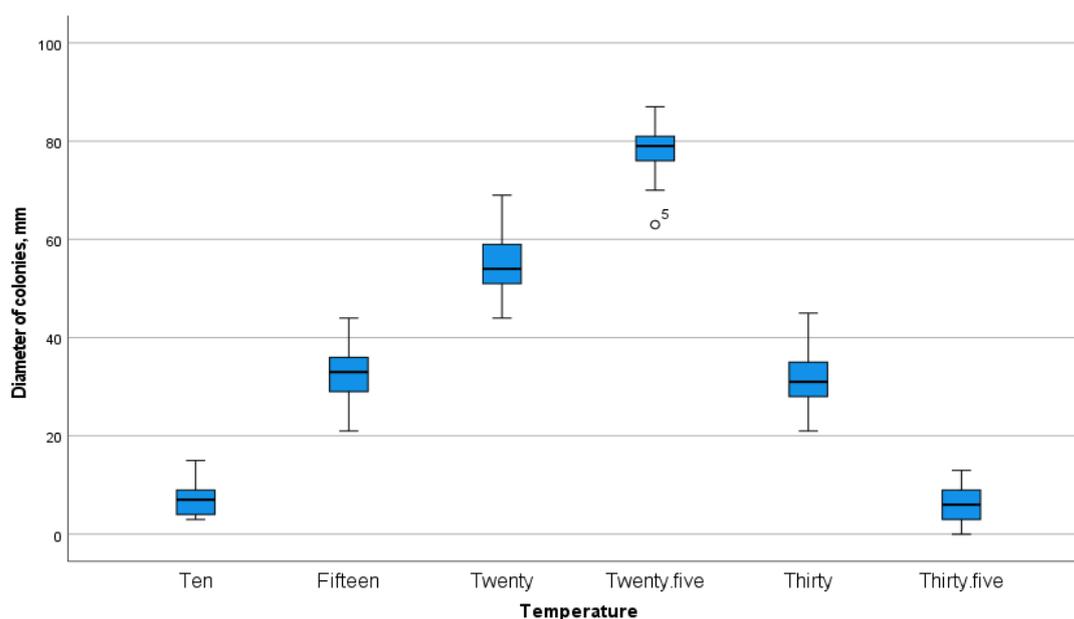

Figure 3. Growth of colonies of natural isolates of EPF isolated from the soils of southern Kazakhstan on Sabouraud nutrient medium on the 29th day after sowing at different air temperatures.

The observations showed that the most favorable temperatures for the growth of entomopathogenic fungal isolates on modified Sabouraud medium were +20°C and +25°C. There were no exceptions among the isolates. However, we were interested in isolates that maintained viability at elevated temperatures. As a result, the promising isolates based on thermotolerance are B13, B14, B15, B22, B23, B24, B25, B23-23, Bc4, Bc7, and Bc8.

Temperature is one of the environmental factors that limits the widespread use of EPF as mycoinsecticides. To mitigate the impact of adverse environmental factors, sodium alginate—a biopolymer that forms a capsule to preserve fungal viability—was used. Microcapsules were obtained by adding droplets of $CaCl_2$ solution (0.25 mL) to liquid sodium alginate. The advantages of using sodium alginate include its slow dissolution in water, forming a gel that withstands temperature changes. Therefore, it is widely used in cosmetics, medicine, and the food industry. Thus, the obtained formulation is environmentally safe for humans.

3.3. Viability of Fungal Conidia at Different Temperature Regimes

The level and rate of conidia germination were determined at two air temperatures, +20°C and +25°C, over three days with 12-hour intervals. Analysis of the obtained data showed high variability in the tested cultures regarding this indicator. The final proportion of germinated spores (after 72 hours) varied between 3.7% and 74.1% on average across the entire sample of cultures (Figure 4).
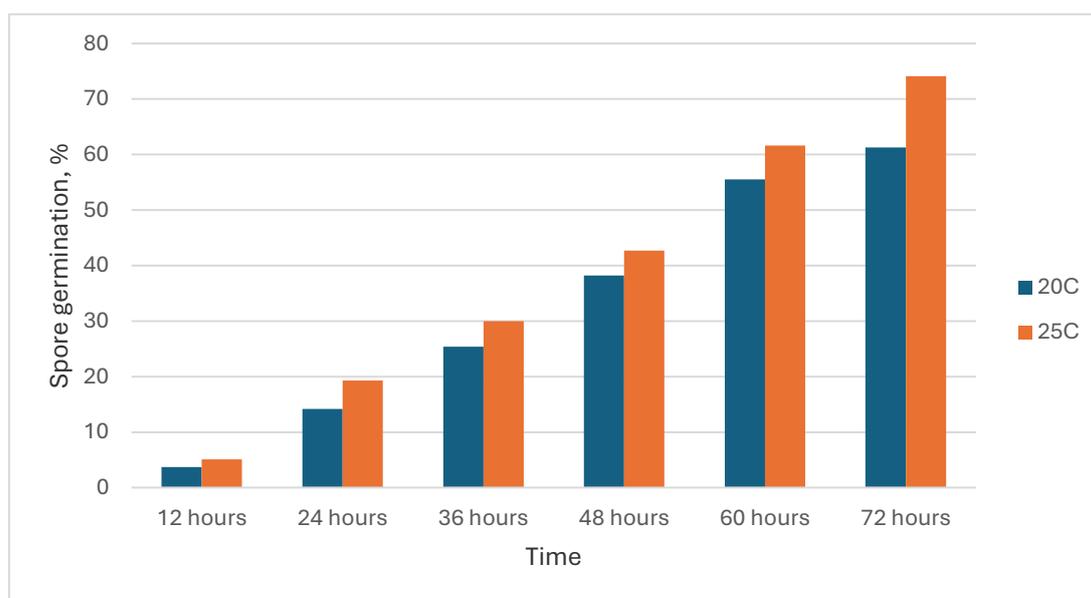
Figure 4. Germination of conidia of natural isolates of EPF isolated from the soils of southern Kazakhstan in a drop of water at different temperatures.

For most of the studied cultures, the most optimal temperature was +25°C for both temperature regimes. However, for several isolates, the germination rate at +20°C and +25°C was higher than that of other cultures, reaching up to 99.1%. Based on the observations, a group of isolates with increased germination rates was identified: B21, B22, B23, B24, B25, B26, B27, B28, B29, B30, Bc3, Bc4, Bc5, Bc7, and Bc8. Some of these isolates not only exhibit increased conidia germination rates but also increased radial growth; thus, they are considered thermotolerant isolates.

3.4. Assessment of the Virulence of Natural Entomopathogenic Fungal Isolates against Colorado Potato Beetle Larvae

An important aspect of evaluating the potential of producer strains is assessing their biological activity. Observations showed significant variability among the studied fungal cultures in terms of virulence (Figure 5). The analysis of the results indicates that the high-virulence group includes the following isolates: B13, B14, B15, B16, B17, B18, B19, B20, B21, B22, B23, B24, B25, B26, B27, B28, B29, B53, B23-2, B23-11, B23-13, B23-14, B23-18, B23-23, B23-25, Bc2, Bc3, Bc4, Bc5, Bc6, Bc7, and Bc8. For these isolates, the final mortality rate (on the 11th day after inoculation) ranged from 90% to 100%. The best mortality rates were observed in isolates B13, B14, B15, B22, B23, B24, B25, B23-23, Bc4, Bc7, and Bc8. The mortality rate after infection with these cultures reached 50-60% on the 7th day and up to 100% on the 11th day, while for other cultures the mortality rate at this time varied from 30% to 40% and reached up to 80-90% on the 11th day.
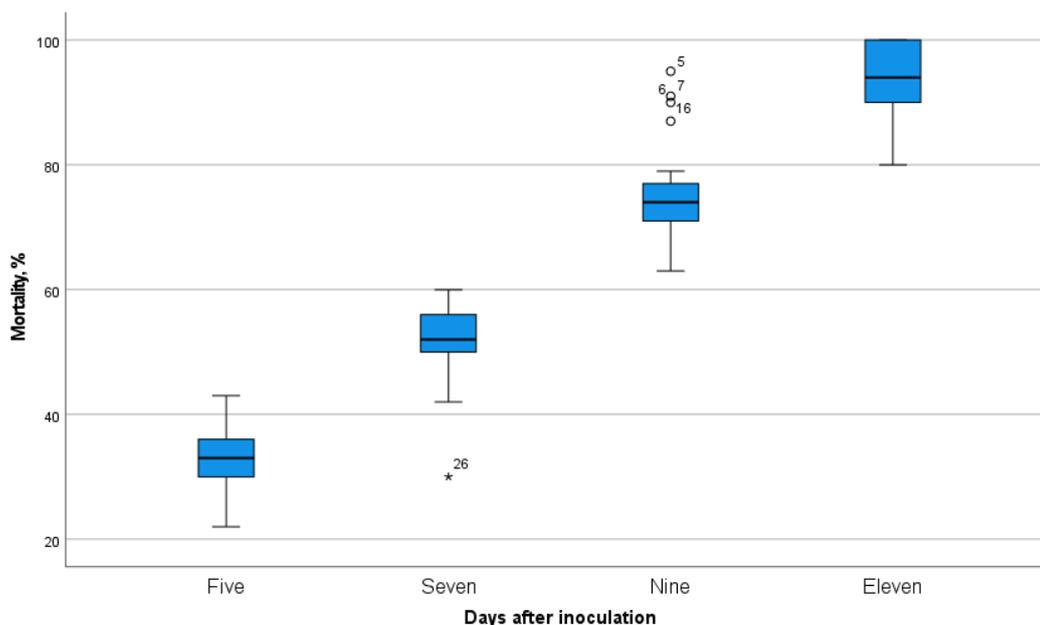
Figure 5. Virulence of natural isolates of EPF isolated from soils of southern Kazakhstan in relation to second- and third-instar larvae of the Colorado potato beetle.

The assessment of fungal colonization of insect cadavers by specific isolates showed a positive correlation (r = 0.88) (Figure 6). This indicates that, as mortality increases, the level of mycosis in Colorado potato beetle larvae also increases. For most isolates, the proportion of dead individuals with clear signs of mycosis exceeded 61%.
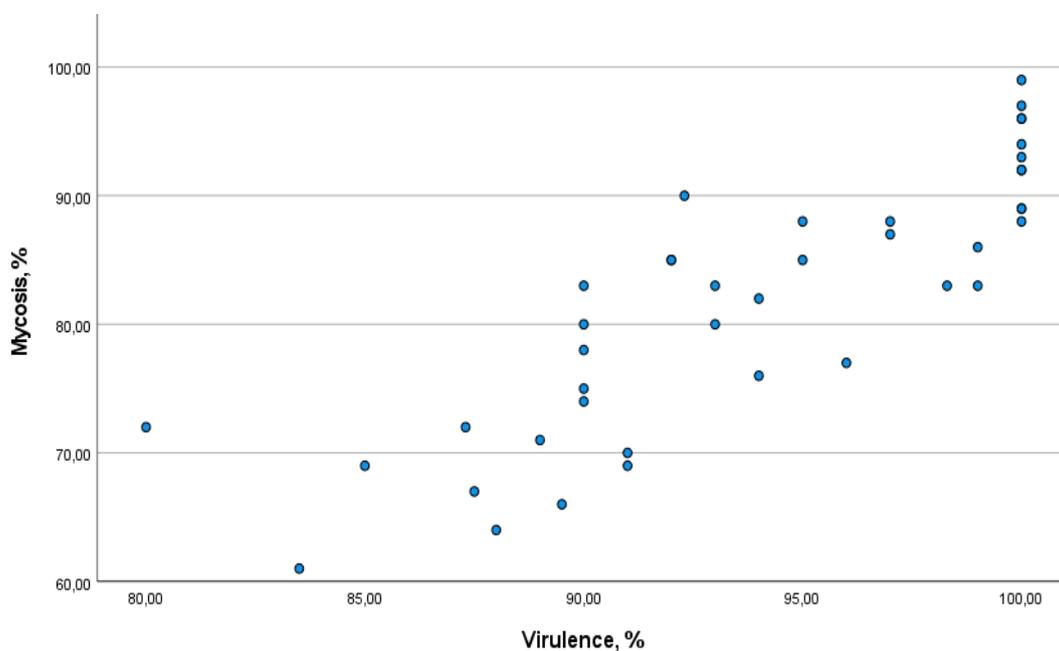

Figure 6. Relationship between the level of mycosis of Colorado potato beetle larvae and the mortality rate.

On average, there was a trend that higher virulence of an isolate was associated with an increased level of mycosis. With 100% virulence, the level of mycosis exceeded 80%. The results indicate high effectiveness of these isolates.

4. Discussion

Climate change, soil degradation, and the indiscriminate use of chemical pesticides and fertilizers pose risks to the sustainability of food and agricultural systems. Specifically, excessive use of chemical insecticides leads to the emergence of resistant pest populations. In this context, EPF have significant potential for controlling Colorado potato beetle populations, as their complex pathogenic process reduces the likelihood of developing resistant populations. Identifying promising EPF strains involves understanding the pathogen, along with assessing the virulence, thermotolerance, and productivity of these strains.

To assess the potential of promising producer strains in continental climates, new isolates from soils in southern Kazakhstan were selected and used for experiments. The results support the notion that soil is a natural habitat for EPF [92–94]. Research indicates that the species richness of EPF in Kazakhstani soils is rather modest. Most of the isolated fungi belong to the genus *Beauveria*, which aligns with the idea that these fungi are widely distributed in nature [95–97]. According to Kazartsev and Lednev, B. pseudobassiana is more common in forest ecosystems, while *B. bassiana* is more frequently found in agricultural areas [98]. Our results are consistent with this theory, as *B. bassiana* predominated in the isolated cultures from the soil. However, previous studies by us showed that *B. bassiana* was more prevalent than B. pseudobassiana in forest ecosystems in the Czech Republic [99]. Therefore, it is not possible to definitively correlate the distribution of a specific fungal species with its geographic origin.

It is known that different fungal strains may exhibit varying degrees of tolerance to limiting abiotic environmental factors, particularly high or low temperatures. Thus, finding strains of EPF with different temperature preferences is relevant from an applied perspective, as high temperatures in continental and arid climates are a major limiting factor for the use of entomopathogenic fungi-based microbial insecticides [100]. In our experiment assessing tolerance to suboptimal temperatures, no significant differences between fungal species were found. This is a topic of considerable debate in the literature. Some authors suggest that EPF can be adapted not only to climatic zones as a whole but also to specific habitats within those zones [61]. Others state that isolates from agroecosystems are more adapted to high temperatures (+37°C), while isolates from forest and arctic regions are adapted to low temperatures (+8°C) [62]. Additionally, Lednev and colleagues report that B. pseudobassiana cultures are inclined towards temperate latitudes and forest landscapes and thus tend to be psychrophilic [101]. In contrast, *B. bassiana* is primarily associated with arid zones and tends to be thermophilic. Moreover, Kryukov and colleagues note that the thermotolerance of fungal isolates significantly increases from north to south [102]. Our results align with these findings, as a group of isolates capable of maintaining viability at high temperatures was identified. However, a general trend of decreased growth rate for isolates at suboptimal temperatures was observed.

To mitigate the impact of negative environmental factors, we decided to use the biopolymer sodium alginate, which is often used for encapsulation and the production of granules and nano- or microspheres containing conidia of entomopathogenic fungi. The literature describes how this polymer can enhance the characteristics of EPF by prolonging conidia survival and activity in field conditions [66–71]. Therefore, further research is planned to evaluate the effectiveness of these microcapsules in field conditions.

A common issue in scientific studies is whether the virulence of EPF depends on the source of isolation or geographic origin. Some authors suggest that the host species from which the EPF were isolated is more important than their geographic origin. They show that EPF isolated from Colorado potato beetle populations are more virulent and promising for pest control compared to strains isolated from other sources [103,104]. Conversely, other authors argue that despite the source of isolation, other entomopathogenic fungal isolates can also be lethal and considered promising for Colorado potato beetle control [105,106]. Our results support the latter view. The isolates we obtained from soils in southern Kazakhstan exhibited high virulence against Colorado potato beetles, with mortality rates ranging from 90% to 100% on the 11th day post-treatment. However, the potential of EPF in Colorado potato beetle control is still not fully understood, and efforts to improve their effectiveness are ongoing. Some studies have even experimented with using EPF in combination with

nematodes to enhance efficacy, though the results are mixed [107,108]. Currently, it is clear that most EPF are highly virulent against Colorado potato beetles regardless of the source of isolation.

5. Conclusions

In this study, 41 isolates of EPF were obtained from soils in southern Kazakhstan to study the effectiveness of EPF in controlling Colorado potato beetle populations. Preliminary identification of isolates was performed using keys. Analysis of the species composition of the isolated cultures based on morphological traits revealed that most isolates belonged to the genus *Beauveria*. PCR analysis was conducted for the ITS and TEF loci to clarify the taxonomic status of natural isolates. PCR analysis of the nuclear DNA locus tef (elongation factor Ef1a) showed that all isolates belong to *B. bassiana*. However, the ITS locus analysis revealed two cryptic species: *B. bassiana* and *B. pseudobassiana*. The majority of isolated cultures were *B. bassiana* – 85% (35 isolates), with *B. pseudobassiana* being the second most common at 15% (6 isolates). No other fungal species were detected. This may be due to the ITS and TEF loci being insufficiently informative. Therefore, further research with other loci (Bloc, RPB1, RPB2, nrSSU, nrLSU) is required.

The optimum temperature for radial growth of natural fungal isolates on Sabouraud medium is between +20°C and +25°C. The highest tolerance to suboptimal temperatures (+15°C and +30°C) was observed in 17 out of the 41 studied cultures. The assessment of conidia germination rates at two temperature regimes, +20°C and +25°C, showed that +25°C was optimal for most isolates. Isolates B21, B22, B23, B24, B25, B26, B27, B28, B29, B30, Bc3, Bc4, Bc5, Bc7, and Bc8 not only exhibited increased conidia germination rates but also increased radial growth, making them the most thermotolerant isolates.

Most natural isolates of EPF exhibited high virulence against Colorado potato beetles, with mortality rates ranging from 90% to 100% on the 11th day post-treatment. However, isolates B13, B14, B15, B22, B23, B24, B25, B23-23, Bc4, Bc7, and Bc8 not only showed high virulence but also high tolerance to suboptimal temperatures. Therefore, they are considered the most promising for use in the sharply changing continental climate of Kazakhstan and in other arid zones.

Author Contributions: R.A.: project leader, main investigator, funding; M.M., Z.A., N.T., B.M., A.K.: formal analysis, statistical processing of data, assistance in methodology and in writing the article; G.A.: isolation of entomopathogenic fungi and laboratory experiments; N.A.: inoculation of larvae Colorado potato beetle and observation. All authors agree with the final version of the article.

Data Availability Statement: All data is presented in this article.

Conflicts of Interest: All authors declare no conflicts of interest.

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