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Impact of *Fusarium* Species Composition and Incidence on Onion Basal Rot in Northeastern Israel

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Abstract: Fusarium basal rot (FBR) places a significant limitation on *Allium* production worldwide. The damage caused by the disease can be observed throughout the entire crop cycle. This research aimed to further our understanding of the impact of FBR on the cultivation of onions (*Allium cepa*) in northeast Israel. It focused on studying the composition and incidence of *Fusarium* species involved in disease outbursts in two representative fields, one in Galilee (Hula Valley) and the second in the Golan Heights, where the disease incidences reached 8%. Using colony morphology, microscopic taxonomic keys, and molecular methods, a new, unreported *Neocosmospora* (previously *Fusarium solani*) species complex (SC, mostly *N. falciformis*) was discovered as a wildly spread member of the *Fusarium* pathobiome community. This species complex appeared more generalist in its nature since it was found in all three onion cultivars' samples. It was also less virulent in seed germination (42–52% higher sprout biomass, $p < 0.05$) and bulb pathogenicity tests (41–45% less necrotic) than *Fusarium acutatum*. Whereas the Galilee yellow Orlando (Riverside) onion cultivar bulbs sampled were colonized by *Neocosmospora* SC (70%) and two other, less abundant species, *F. oxysporum* f. sp. *cepae* and *F. acutatum* (15% each), the Golan Heights field's *Fusarium* community showed host specificity. In the Golan Heights field, *F. oxysporum* f. sp. *cepae* inhabited the red Ha2 onion cultivar bulbs, whereas *F. acutatum* colonized the yellow Ha1 cultivar (40% and 50% prevalence along with *Neocosmospora* SC). A better understanding of the complexity of this disease caused by different *Fusarium* species and with a divergence in host susceptibility and virulence is critical for developing disease management strategies. Since each *Fusarium* species reacts differently to pest control treatments, changes in the species composition may require specifically adapted management solutions.

Keywords: *Allium cepa*; basal rot; distribution; fungus; *Fusarium*; onion; pathogenicity assay; PCR

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1. Introduction

Onion (*Allium cepa* L.) is an important agricultural crop globally. In 2022, the global planted area of onions and shallots was estimated to be 5,967,491 ha, representing a production of ca. 110,616,270 tons of dry cultivars (FAO 2022, available at: https://www.fao.org/faostat/en/#data/QCL, accessed 1 April 2024). Fusarium basal rot (FBR, also known as Fusarium rot, Fusarium wilt, or basal plate rot of *Allium* spp.) is a serious fungal disease affecting onion crops in many parts of the world [1]. Notwithstanding the ubiquitous incidence of FBR across various *Allium* species, knowledge pertaining to this disease remains fragmented and incomplete [2].

The FBR disease is caused by various species of the genus *Fusarium*, with *Fusarium oxysporum* f. sp. *cepae* (formae speciales in *Allium cepa*) being the most commonly reported species [2]. The pathogen infects the onion plant through its roots and causes rotting of the basal plate (the roots and stem connection portion). Infected plants may show delayed growth, yellowing, and wilting. Young seedlings and dormant mature onion

bulbs are the two most valuable plant phenological stages of FBR. Yet, the disease can occur throughout the entire crop cycle [3–6]. It leads to seedlings' pre- and post-emergence mortality (damping off) and can cause significant crop losses. Yield losses in the field can reach 39% (Turkey [7]) and 50% (Nigeria [8]), depending on the inoculum pressure and the cultivar's susceptibility. FBR pre- and post-harvest results in significant crop reduction due to reduced bulb size (growth suppression), bulb rot, and decreased onion shelf-life [9].

Interspecies variation in virulence is a common feature in *Fusarium* populations, and its impact on the developmental stage susceptibility to FBR is well-documented [10]. In most cases, onion susceptibility to FBR decreases with seedling age but increases at bulb development until post-harvest [5]. Nowadays, studies are attempting to uncover the mechanisms behind those differential responses and the role of mycotoxins in determining disease outcomes. These are particularly important knowledge gains since the *Fusarium* species produce various mycotoxins that may harm human health and animals [11]. Defense-related genes in seedlings and bulbs are expressed depending on the *Fusarium* isolates' aggressiveness as part of the plant defense [1]. Yet, in susceptible plants, they may not be effective in ensuring a resistance response against FBR. Meanwhile, secondary fungal metabolites play a differential role during colonization at the respective stages. For example, the toxin fumonisin B1 appears to be a virulence factor specific to the seedling phase [1].

Notwithstanding the extensive implementation of various control measures, the disease continues to pose a significant challenge for *Allium* producers on a global scale [2]. Early detection and rapid disease management action are essential to minimize the impact on onion crops. FBR control toolkits should utilize a combination of cultural, chemical, and biological control measures. These could include planting disease-free onion sets, using crop rotation to reduce the buildup of the fungus in the soil, applying fungicides, and using biocontrol agents such as *Trichoderma* and *Bacillus* [2]. In addition, it is crucial to apply good sanitation practices to prevent the spread of fungus. Such hygiene practices could include removing infected plants and crop residuals from the field and disinfecting tools and equipment between uses.

Recent research has also explored other control strategies, such as breeding for resistance and RNA interference (RNAi) to silence specific fungal genes [12,13]. For example, it was demonstrated that spray application of a long dsRNA (791 nt CYP3-dsRNA), that targets *F. graminearum* cytochrome P450, lanosterol, and C-14α-demethylase genes (required for fungal ergosterol biosynthesis) significantly inhibited fungal growth [14]. Furthermore, synthetic siRNAs proved to down-regulate key fungal genes involved in *Fusarium* toxin production [15,16]. In banana (*Musa* sp.), significant resistance (70–85% reduction in disease symptom) to *F. oxysporum* was observed at eight months post-inoculation in the RNAi plants that could silence the *Fusarium* velvet protein complex (which regulates fungal development and secondary metabolism) and transcription factor 1 gene [17].

In 2022, onion and shallot bulb (dry, excluded dehydrated) production in Israel covered an area of 3865 ha throughout the country, and the commercial production reached 82,503 tons (FAO 2022, available at: https://www.fao.org/faostat/en/#data/QCL, accessed 1 April 2024). Fusarium basal rot has been reported as a significant disease of onion crops in Israel, with outbreaks occurring in various onion-growing regions in the country [18,19]. Despite the latest scientific efforts [9,20–22], information regarding Israel's FBR prevalence is scarce. In a recent study, four distinct *Fusarium* species were successfully isolated from onion bulbs sampled from infected fields in northeastern Israel's Golan Heights region [21]. The isolated species, *F. proliferatum*, *F. oxysporum* f. sp. *cepae*, *F. acutatum*, and *F. anthophilium*, were identified and characterized. The latter two species are lesser-known species implicated as FBR causal agents. Despite these findings, other pathogenic *Fusarium* species may also contribute to FBR. Significant knowledge gaps remain pertaining to the nature and distribution of the disease in Israel and the control measures employed to combat it. Specifically, no structured data have been reported concerning the disease's historical prevalence or its rate of spread over time, and there is no current map of its

distribution. Furthermore, no onion cultivars demonstrating resistance to FBR have been identified. Only recently have fungicides that can effectively target the disease's causal pathogens been established [9], and their application on a commercial field scale has not yet been accomplished.

The objectives of the present study were to improve our understanding of the *Fusarium* species involved in FBR epidemics in two representative commercial fields in northeastern Israel, one in Galilee (Hula Valley) and the other in the Golan Heights. Based on the accumulating scientific data [9,20–22], our research hypothesis was that the variety of *Fusarium* species involved in FBR would be found to be much greater than that so far discovered and that they would be found to thrive in complex compositions depending on the host plant and the environment. We also hypothesized that the impact of the disease in some commercial fields would be found to be more significant than previously thought. The disease damage was evaluated in a field survey at the flowering stage by determining the dry inflorescence percentage in a sample of 2000 plants. Fungal species were isolated from bulb samples and identified using colony morphology, microscopic evaluation, and molecular targeting of the *Fusarium* translation elongation factor-1 alpha gene (TEF1), the RNA polymerase largest or second-largest subunit (RPB1 or RPB2), and the *F. oxysporum* f.sp. *cepae* species-specific putative effector secreted in xylem genes 3 (SIX3). Using a phylogenetic tree and the inter simple sequence repeat (ISSR)-polymerase chain reaction (PCR) molecular method, the *Fusarium* microflora of each onion cultivar was uncovered, leading to a curious new insight. The newly identified *Neocosmospora* (previously *F. solani*) species complex (SC) was tested for its pathogenicity towards onion seedlings and bulbs, and Koch's postulates were accomplished. The results of this study provide deep insights into the pathosystem associated with Fusarium basal rot and its significant implications for disease management decision-making.

2. Materials and Methods

2.1. Evaluation of Disease Severity

This study aimed to enhance our understanding of FBR in onion cultivars infected with toxigenic *Fusarium* species in northeastern Israel. The FBR disease incidence was evaluated in a commercial field in northeast Israel's Golan Heights, part of the Givat Yoav farm, termed plot 8 (32°48′16.7″ N 35°40′16.2″ E, Figure 1). The area was sown on 7 October 2021, and sampling was carried out on day 237 from sowing (1 June 2022). We utilized our fieldwork to sample onion bulbs for the Fusarium species identification study and conducted a disease incidence survey, although this was not the primary focus of the current work. The Givat Yoav field, which was included in the survey, consisted of inbred plants that had undergone vernalization and were in the flowering stage. This enabled us to easily identify diseased dry inflorescences and conduct the survey without reducing crop yield. In contrast, the second field, Yiron's field, was seeded with a hybrid yellow Orlando (Riverside) cultivar and was cultivated for bulb production, thus denying the flowering stage. Consequently, identifying diseased bulbs near the end of the season (before harvest) in 2000 plants could have reduced the yield, and we decided to avoid this. Moreover, the different cultivation purposes (seeds or bulbs) rendered these two fields noncomparable.

The FBR assessment survey was conducted in the eastern part of the plot. Along the 300 m row, ten inflorescences were counted at every two meters. Each blossom was gently drawn. The diseased plants were easily uprooted. In the red onion varieties (Ha2—a male breed cultivar, and Ha3—a female cultivar), 500 inflorescences were sampled per cultivar. In the yellow variety (Ha1—a female cultivar), 1000 inflorescences were tested. All three cultivars were from Hazera Seeds Ltd. (Berurim, Israel). The disease incidence (number of diseased plants/total plants sampled) was calculated according to [21].

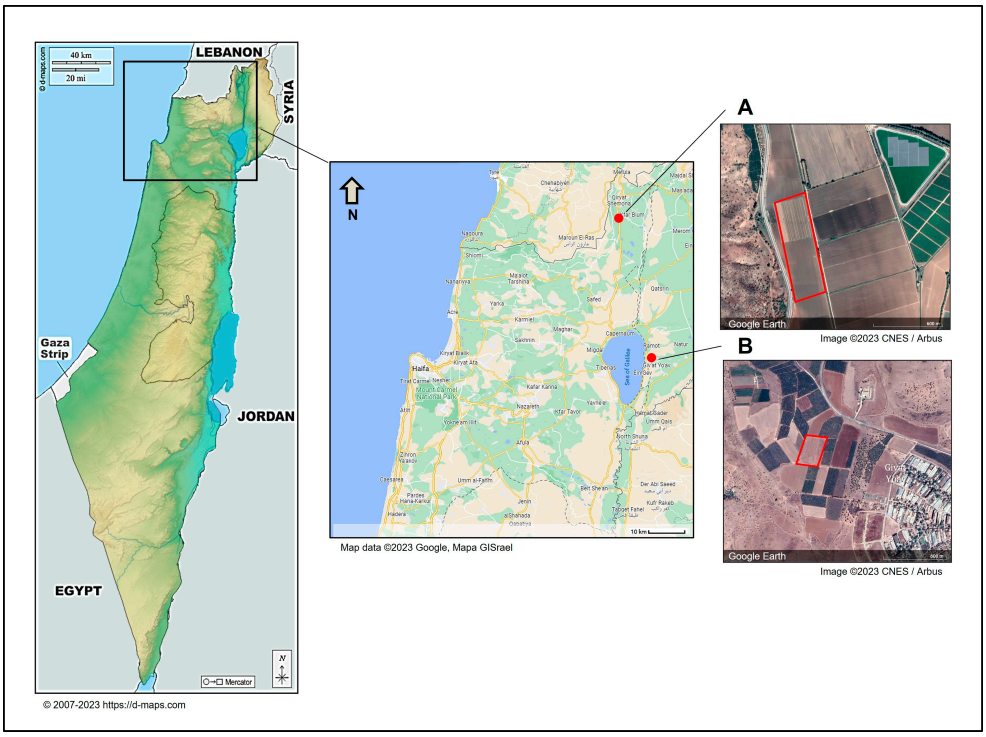

Figure 1. The locations of sampling sites in northeastern Israel. Onion samples were collected from two representative commercial fields in northeast Israel. The sampled fields were Kibbutz Yiron (Galilee, Hula Valley, (**A**)) and Givat Yoav (Golan Heights, (**B**)), highlighted by red boxes. The Givat Yoav field was also used for a survey evaluating disease severity. According to d-maps.com (accessed on 1 April 2024) and the Google Maps/Google Earth (https://www.google.com/intl/en_GB/help/terms_maps/, accessed on 1 April 2024) Terms of Service, the maps and photos can be used and adapted. The map of Israel is from https://d-maps.com/pays.php?num_pay=88&lang=en (accessed on 1 April 2024).

2.2. Isolation of Pathogens from Diseased Onion Plants

Near the time of onion collection (the season's end), onion samples were collected from two representative commercial fields of onion production in northeast Israel (Galilee and Golan Heights). The two fields were typical of Israel's northeast agriculture region. The tested fields were Givat Yoav and Yiron (Figure 1). Yiron's field (33°09′19.4″ N 35°34′23.1″ E) was sown with the yellow Orlando cv. on 9 January 2022 and was sampled on day 228 of sowing (25 August 2022). The Givat Yoav field (described in Section 2.1) included three onion cultivars: red female (Ha3 cv.), red male (Ha2 cv.), and yellow female (Ha1 cv.) A total of 67 onion bulbs were sampled on day 237 from sowing (1 June 2022). About 20 samples were taken arbitrarily from each onion variety grown in the Sde Yoav and Yiron fields to isolate possible FBR causal agents. These samples included healthy-looking onions (about one-third of the bulbs) and infected onions. Infected onion plants had typical FBR-characteristic symptoms of discoloration of the roots to brown, rotting in the basal plate (change in color to brown and the appearance of moist or dry necrotic areas), and separation of the scales (fleshy leaves). Some of the samples were infected by secondary parasites, such as maggots of the onion fly (*Delia antiqua*).

For the pathogens' isolation, the onion's basal plate was cut from each bulb, about 7 mm from the lower tip (all roots, if present, were removed). Each basal plate was divided into 3–4 pieces of tissue. All the pieces were disinfected with 3.5% commercial bleach (NaOCl) for about 30 s and then thoroughly washed for 1 min with tap water. Each tissue was placed in the center of a 90 mm Petri dish that contained potato dextrose agar (PDA). The PDA medium was made by dissolving 39 g of PDA powder in 1 L of double-distilled water (DDW).

The isolates (228 plates in total) were incubated in the dark at 28 ± 1 °C. After two days, the developed isolates were transferred to new PDA media. Transfer to new plates was performed for isolates with colony characteristics and spores corresponding to the *Fusarium* genus, and the process continued until pure colonies were obtained. The series of transformations for each colony to a new plate was performed each time by taking a colony agar disk from the young margins of the old colony and carefully tracking the colony morphology. Additionally, molecular verification using the ISSR and sequencing was performed to ensure that a single species was obtained.

The isolates were classified according to their colony characteristics. Of these, 31 were selected according to their morphological and spore characteristics resembling the *Fusarium* genus and subjected to molecular identification (PCR, gel electrophoresis, sequence determination, and homology search against the GenBank and the Fusarioid-ID databases).

2.3. DNA Extraction and Molecular Identification of Fusarium spp.

Molecular identification was conducted for each of the *Fusarium* species involved, according to the methods described previously [21]. DNA extraction was performed using the Master Pure™ Yeast DNA Purification Kit (Epicentre, Madison, WI, USA). The DNA concentration and purity test after extraction was carried out using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) via the microvolume spectrophotometric UV–vis method. The test ensures a high DNA quality (median value of 1.8 or above of absorbance ratio at wavelength 260/280 nm) and at least 30 nanograms/µL DNA. The samples' average DNA concentration was 83 nanograms/µL. Molecular identification (PCR followed by sequencing) was performed using the Fa/R8 or 7cF/11aR primers targeting RPB1 or RPB2—the RNA polymerase largest or second-largest subunit (Table 1). Additionally, we used the primers E1/E2 (specific to the genus *Fusarium*, targeting the *Fusarium* translation elongation factor-1 alpha gene, TEF1) (Table 1). DNA fragments were amplified by PCR and detected by gel electrophoresis.

The PCR was conducted using the T-100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with a total volume of 25 µL per reaction. Each of the reaction mixtures tested here included 1 µL of a primer mix (each primer in the solution was at a concentration of 10 µM), 12.5 µL of the commercial reaction mixture (PCRBIO Hot Start VeriFi™ or PCRBIO Taq Mix Red, PCR Biosystems, London, UK), 1 µL of template DNA, and 10.5 µL of UltraPure water (Bio-Lab, Jerusalem, Israel). For the TEF1 gene amplification, the PCR conditions consisted of an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. For the RPB1 or RPB2 gene amplification, the PCR protocol was initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

Microsatellite-sequence-based primers were used for the ISSR-PCR molecular technique (Table 1). This approach generates multilocus markers for DNA fingerprinting [23]. PCR reactions for ISSR amplification were conducted with the following parameters: an initial denaturation step at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 43 or 50 °C for 15 s, and extension at 72 °C for 30 s. The samples were then cooled to 12 °C for sample retrieval.

Table 1. Primers used for the *Fusarium* spp. detection.

Primer	Gene	Sequence [a]	Fragment Length	Reference
E1/E2	TEF1—*Fusarium*-specific	F-ATGGGTAAGGAGGACAAG	680 bp	[24]
		R-GGAAGTACCAGTGATCAT		
7cF/11aR	RPB2—RNA polymerase second-largest subunit	F-ATGGGYAARCAAGCYATGGG	~970 bp	[25]
		R-GCRTGGATCTTRTCRTCSACC		

Table 1. *Cont.*

Primer	Gene	Sequence [a]	Fragment Length	Reference
Fa/R8	RPB1—RNA polymerase largest subunit	F-CAYAARGARTCYATGATGGGWC	1607 bp	[26]
		R-CAATGAGACCTTCTCGACCAGC		
SIX3 F/R	*Fusarium oxysporum* f. sp. *cepae* secreted in xylem genes 3	F-ATGCGTTTCCTTCTGCTTATC	306 bp	[21]
		R-AGGTGCGACATCAATGACAG		
ISSR1	Inter simple sequence repeat	F + R-AGAGAGAGAGAGAGA	Multiple lengths	[23]

[a] Y = C or T, R = A or G.

2.4. Identification of the Fusarium Species and Phylogenetic Relationships

A total of 31 isolates amplified with TEF1 or the RPB1/RPB2 genes were sent for sequencing (Macrogen Europe, Amsterdam, The Netherlands). Similarity percentages between sequences compared to other already recognized species were determined online using the identification database Fusarioid-ID (accessible at www.fusarium.org, accessed on 25 March 2024) and an NCBI GenBank BLASTN search (National Center for Biotechnology Information, Bethesda, MD, USA, at: http://www.ncbi.nlm.nih.gov, accessed on 25 March 2024). Sequence comparison of the TEF1 gene-conserved regions was conducted using the Clone Manager 11.0 program (Sci Ed Software, Durham, NC, USA). Sequences were aligned, and phylogenetic tree construction was performed using the SeaView version 5.0 software (http://doua.prabi.fr/software/seaview, accessed on 25 March 2024) [27]. The trees were constructed using a distance-based method with the default parameters (BioNJ (neighbor-joining algorithm), distance (maximum likelihood), Jukes–Cantor (J-C), ignore positions with gaps, and bootstrap based on 1000 tree replications). The phylogenetic trees that presented similarity percentages between sequences, were generated with the TEF1 Fusarium-specific sequences. The analysis also used the TEF1 gene from reference strains to assist with taxonomic assignment. These included previously identified *Fusarium* species in Israel's Golan Heights (isolates B1, B5, B7, B8, B14, and B16 [21]) and four reference *Neocosmospora* (*F. solani*) SC species that were taken from the GenBank: isolate cc41W (HQ731052.1), strain hc0001 (KP143718.1), isolate DB-C2 (KY486693.1), and strain gss53 (MH341207.1). Also, an outgroup was set using the onion pathogen *Rhizopus arrhizus* (E12 strain, MK174988.1), isolated and identified as part of the current research.

2.5. Colony Morphology and Identification of the Fusarium Species

The *Fusarium* species involved in FBR were isolated from onion bulbs and identified using the microscopic characteristics of the spores and colony morphology, according to [21]. All isolates were grown on PDA plates for four days to allow hyphae and spore formation. Mycelial mats or conidia were carefully scraped off the plates, and a small amount was suspended in 10 µL of potato dextrose broth (PDB) or DDW. The suspensions were then placed on sterile glass slides for microscopic observations using a light microscope at a magnification of 250× without staining.

2.6. Germination Pathogenicity Assay

The pathogenicity test was conducted according to [21] with modifications and aimed at assessing the virulence level of the *Neocosmospora* SC isolates on onion seedlings. The experiment was performed with four replicates using Petri dishes, each containing ten onion seeds. The two onion cultivars selected for this seedling test (and the bulb assay described below) were the yellow onion cultivar Orlando and the red onion cultivar Noam (supplied by Hazera Seeds Ltd., Berurim, Israel). These varieties were selected because they are widely grown in Israel and are common in the markets. The yellow Orlando cv. is the same as the one sampled from the Kibbutz Yiron (Galilee, Hula Valley) field, and the red Noam cv. is very similar in its characteristics to the red cultivars tested in the Givat Yoav (Golan Heights) field. Seeds were washed to remove their commercial coating (Thiram,

Captan, Carboxin, Metalaxyl-M, manufactured by Rogers/Syngenta Seeds, Boise, ID, USA, supplied by CTS, Tel Aviv, Israel). This procedure was carried out by dipping them in tap water for 15 min and then replacing the water ten times. Seeds were disinfected with 70% ethanol for 1 min, rinsed three times with DDW, soaked for 3 min in disinfection solution (2% NaOCl, 12 µL dish soap, 150 mL tap water), and washed vigorously six times with DDW.

For this seedling assay and the following bulb test, six of the *Neocosmospora* SC isolates were selected (Nos. E3, E7, E8, E9, E14, and E21). These isolates were tested against a non-infected control group and a positive control group, which was inoculated with *F. acutatum* (isolate B5 [21]). After drying the seeds on a sterile paper towel, each group of onion seeds was transferred onto a Petri dish with sterile Whatman paper soaked in sterile DDW. A 6 mm diameter disc was cut from a selected 5-day-old *Fusarium* sp. colony (grown previously on PDA in the dark at 28 ± 1 °C) and placed onto each onion seed group plate (in the middle). The control group was grown without inoculation. After nine days of incubation under gentle rotation in a rotary shaker (to assist hyphae and spores' dispersion) in the dark at 28 ± 1 °C, the seeds were photographed and washed, and their germination percentages, biomasses, and the epicotyl emergence numbers were measured and compared to the mock uninfected control group. A germinating seed was defined as one in which the radicle had broken the seed coat.

2.7. Onion Bulb Pathogenicity Assay

An onion bulb pathogenicity assay was conducted on two cultivars, Orlando cv. and Noam cv. (yellow and red onions), as previously described [21] but with modifications. The experiment included five repetitions per isolate (40 bulbs per cultivar). The same six *Neocosmospora* SC isolates (Nos. E3, E7, E8, E9, E14, and E21) tested in the seed assay were evaluated here. As in the seedling assay, these isolates were tested against a non-infected control and a positive control group inoculated with *F. acutatum* (isolate B5) [21]. The experiment was performed in a sterile environment inside a biological hood. A stock of ca. 2×10^6 spores/mL (in sterile water) was prepared from five-day-old colonies, previously grown on PDA at 28 ± 1 °C in the dark.

The bulbs were sterilized in 70% ethanol for 1 min, and the outer scales were removed. A sterile pipette tip (10 mm in diameter) was used to stab the basal plate once, and 50 µL spores were pipetted into each puncture. For control bulbs, a similar volume of sterile water was injected. Every bulb was stored separately within a sealed sterilized plastic bag to maintain a moist environment and prevent contamination. The bulbs were incubated in a temperature-controlled incubator in the dark at 22 ± 1 °C for two weeks. The appearance of early decay symptoms two weeks post-infection on the bulbs' basal plate exterior and interior tissue and mycelial growth emergence on the bulb surface were assessed. The necrotic lesion dimension was measured as the length from an onion's lower (root) tip to the scales. The necrotic lesion severity was evaluated using categories where 5 indicates severe rotting and 1 is healthy tissue. Finally, the fungus from selected infected onions was re-isolated on PDA and identified to fulfill Koch's postulates.

2.8. Statistical Analysis

The seedlings' and bulbs' pathogenicity assay data were analyzed using Microsoft Excel (Microsoft 365 MSO, version 2401 Build 16.0.17231.20290) and GraphPad Prism software, version 9.5.1.733 (GraphPad Software Inc., San Diego, CA, USA). The data were analyzed using a one-way analysis of variance (ANOVA), the Brown–Forsythe test, and a posterior Dunnett's test (which is restricted to comparing the experimental groups against a single control group) at a significance level of $p < 0.05$.

3. Results

3.1. Evaluation of Disease Incidence

The Givat Yoav field (Golan Heights) was chosen for the FBR field's crop loss estimation. Typical symptoms in red (variety Ha3) and yellow (variety Ha1) female plants were documented (Figure 2). The estimated incidence in the field is shown in Table 2. The disease prevalence in the red onion variety ranged from 2.4% in the male (Ha2 cv.) plants to 8% in the female (Ha3 cv.) plants. For comparison, in female plants of the yellow onion (variety Ha1), an infection level of 2.4% was determined. The red female (Ha3 cv.) plants had statistically significantly higher FBR incidence than the other two varieties ($p < 0.05$).

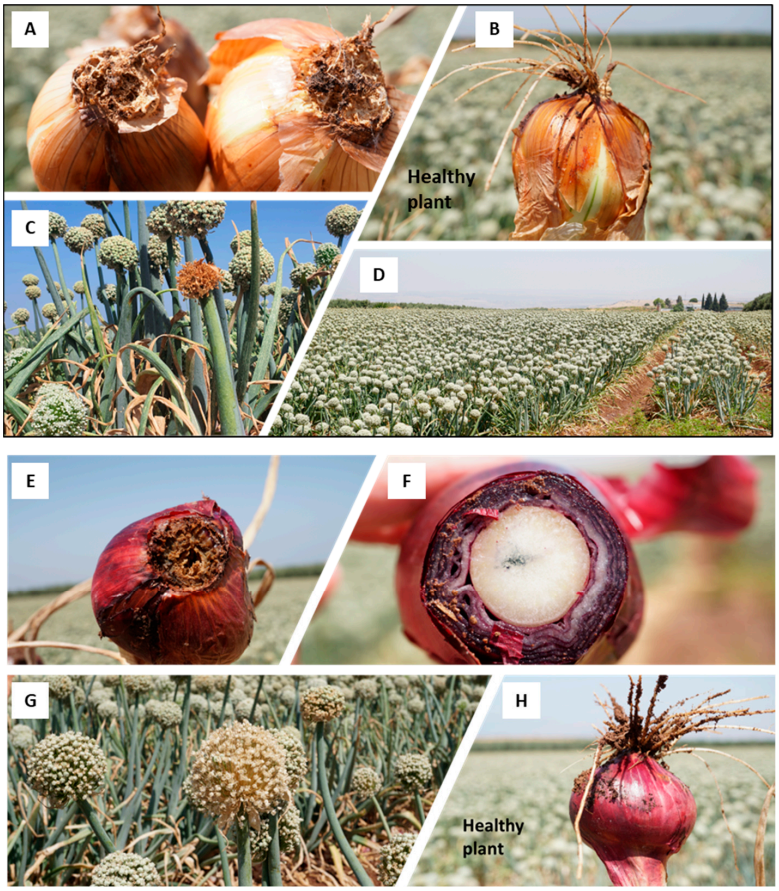

Figure 2. The *Allium cepa* Fusarium basal rot (FBR) disease outcome in the yellow onion (Ha1 cv., (**A**–**D**)) and the red onion (Ha3 cv., (**E**–**H**)) in the Givat Yoav commercial field in northeastern Israel's Golan Heights (location in Figure 1). (**A**,**E**,**F**)—bulb samples with typical FBR disease symptoms. (**B**,**H**)—a healthy-looking bulb sample. (**C**,**G**)—dehydrated yellowish-to-brown color inflorescences scattered in the field. (**D**)—an overview of the field.

Table 2. Disease incidence in the Givat Yoav field, Golan Heights [a].

Cultivar	Number of Inflorescences Sampled	Number of Diseased Plants	Percentage of Diseased Plants [b]
Red, female (Ha3 cv.)	500	40	8% ± 0.22 [c]
Red, male (Ha2 cv.)	500	12	2.4% ± 0.10
Yellow, female (Ha1 cv.)	1000	24	2.4% ± 0.13
Average	-	25.3	5% ± 0.77

[a] Givat Yoav farm, plot 8 (32°48′16.7″ N 35°40′16.2″ E, Figure 1). [b] Values ± standard deviation. [c] Statistically significantly higher disease incidence than in the other two varieties (Z test for two proportions, $p < 0.05$).

3.2. Isolation and Identification of the Fusarium Species from the Collected Onions

About 20 samples (onion bulbs) were taken from each of the onion varieties grown in the Givat Yoav and Kibbutz Yiron fields (Figure 1) to isolate possible disease agents. The onions were in different degrees of decay, and secondary parasites were seen in some of them, such as maggots of the onion fly. In addition, ten onions that looked healthy were sampled from the plants. A total of 67 onions were sampled. From the collected onions, 228 fungal isolates were isolated. Of these, 31 were selected according to their microscopic (spores characteristics) and colony morphological traits resembling the *Fusarium* genus (Figure 3).

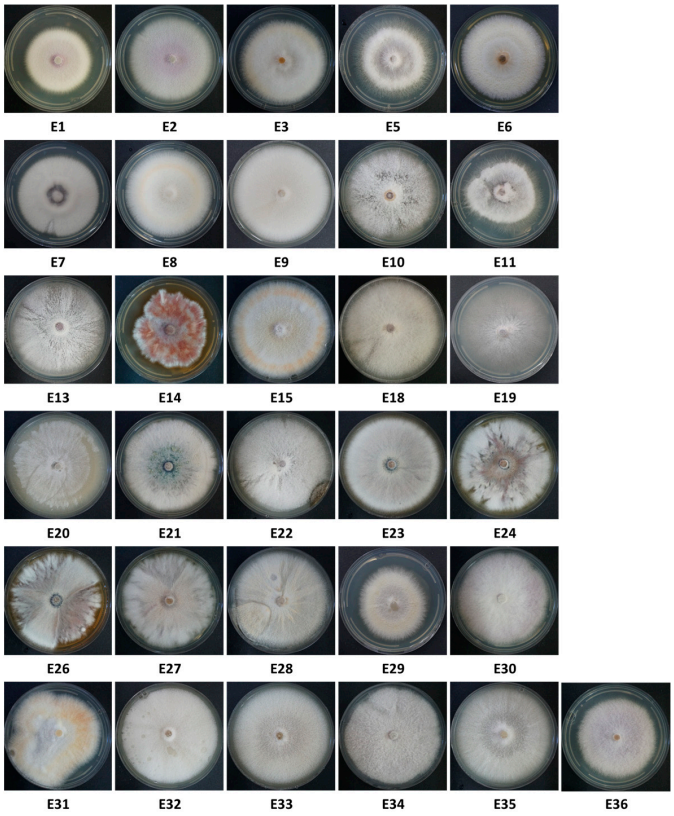

Figure 3. Thirty-one *Fusarium* isolate colonies from onions collected from the Givat Yoav (Golan Heights, E1–E11) and Kibbutz Yiron (Galilee, Hula Valley, E13–E36) fields (see Figure 1). Isolates were selected from 228 according to colony and spore characteristics. Some isolate numbers are missing because they were identified as different species than *Fusarium*. That is why 31 isolates are presented, while some have a higher number (E32–E36). Colonies were grown in the dark for six days on a rich solid medium (PDA) at 28 ± 1 °C.

The sequenced isolates were compared to the Fusarioid-ID (Table 3) and GenBank (NCBI, nucleotide blast BLASTN, Supplementary file—Table S1) databases. The Fusarium Pairwise ID alignment search resulted in a high similarity (>99% in most cases) of the new sequences to *Fusarium* species in this GenBank. Likewise, BLASTN identification results in all analyzed species showed a significant similarity (ranging from 99.10% to 99.98%, except for E3, which reached 91.02%) to the previously reported *Fusarium* spp. sequences in the GenBank database. Interestingly, the new analysis using the Fusarioid-ID database and the phylogenetic analysis described below confirmed that isolate B16, previously thought to be *F. anthophilium* [21], is actually *F. acutatum*. The identity of all other isolates of that previous work was confirmed. The B1 isolate (*F. proliferatum*) is named by the synonym *F. annulatum*.

Final verification of the *Fusarium* species identity was performed by amplifying the *F. oxysporum* f. sp. *cepae* secreted in xylem genes 3 (Table 1, Figure 4). Identification of the *Fusarium* isolates was further reinforced by assessing the similarities between the

isolates using the inter simple sequence repeat (ISSR)-PCR molecular technique. The DNA fingerprinting analysis revealed that isolates of the same species exhibited highly similar DNA profiles (Figure 5). Also, some species were subdivided into subspecies families according to shared homology. The species found included *F. oxysporum* f. sp. *cepae* and *F. acutatum*, which were already found in a previous study [21], but also a new species complex that has to date not been identified—*Neocosmospora* (previously the *F. solani*). The last is dominated mainly by *N. falciformis.* Intriguingly, as detailed below, this species complex was much more prevalent in the isolates' samples than the other two species.

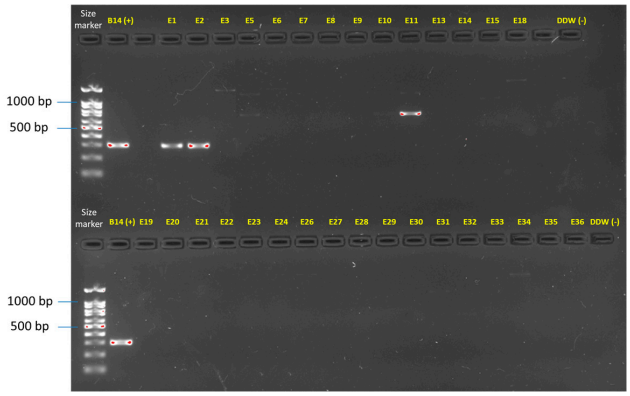

Figure 4. Molecular identification of the *F. oxysporum* f. sp. *cepae* secreted in xylem genes 3 (SIX3, primer sets and references in Table 1). The gel presented is uncropped. This analysis was performed on all the *Fusarium* isolates (detailed in Table 3). Only two isolates (E1 and E2) were identified using this specific approach as *F. oxysporum* f. sp. *cepae*. Isolate E11 and some other isolates present an unspecific band product. The *F. oxysporum* f. sp. *cepae* (isolate B14, [21]) was used as a positive control. Sterile double-distilled water (DDW) was used as a template in the PCR reaction to provide a negative control.

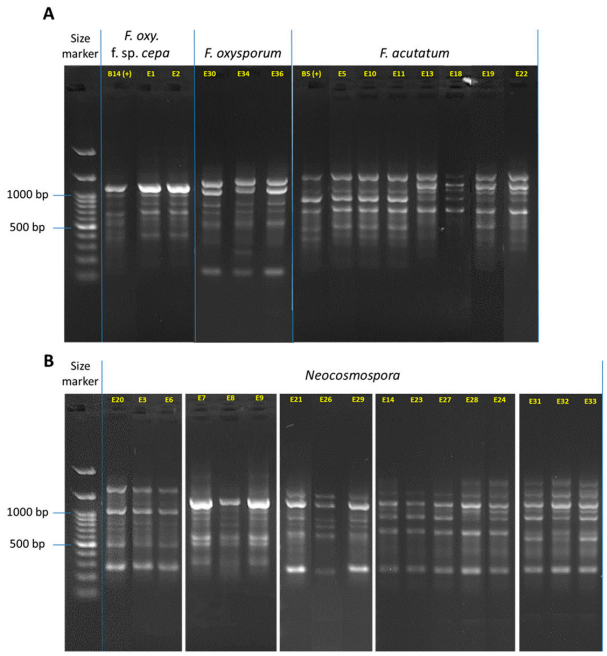

Figure 5. DNA fingerprinting for the *Fusarium* isolates (detailed in Table 3). The complex band profile was generated using the inter simple sequence repeat (ISSR)-PCR molecular method. The ISSR primers are listed in Table 1. The gels were cropped and rearranged to improve the presentation's clarity and conciseness. Full-length gels are presented in Supplementary Figure S2. (**A**) *F. oxysporum* f. sp. *cepae*, *F. oxysporum* species complex (SC, *F. inflexum*), and *F. acutatum* (*F. fujikuroi* SC). (**B**) *Neocosmospora* (*F. solani*) SC. The *F. oxysporum* f. sp. *cepae* (isolate B14, [21]) was used as a positive control.

Table 3. FUSARIOID-ID database identification of the Fusarium isolates from this study [a].

Isolate	Primer	*Fusarium* spp.	Gene	Accession		Similarity	Overlap	Onion Cultivar	Collection Site [c]
E1 [b]	E1/E2	*Fusarium nirenbergiae*, *F. oxysporum* SC [d]	*TEF1/2*	JW 124027	https://www.fusarium.org/details/23/1257	99.85%	98.52%	Ha2	Givat Yoav, Golan Heights
	7cf/11ar	*Fusarium nirenbergiae*, *F. oxysporum* SC	*RPB2*	LC13757	https://www.fusarium.org/details/23/1895	99.88%	77.20%		
E2 [b]	E1/E2	*Fusarium nirenbergiae*, *F. oxysporum* SC	*TEF1/2*	JW 124027	https://www.fusarium.org/details/23/1257	99.85%	98.37%	Ha2	Givat Yoav, Golan Heights
	7cf/11ar	*Fusarium nirenbergiae*, *F. oxysporum* SC	*RPB2*	LC13757	https://www.fusarium.org/details/23/1895	99.64%	85.73%		
E3	E1/E2	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 32544	https://www.fusarium.org/details/23/492	99.70%	91.39%	Ha1	Givat Yoav, Golan Heights
	7cf/11ar	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 475.67	https://www.fusarium.org/details/23/478	99.47%	63.83%		
E5	E1/E2	*Fusarium acutatum*, *F. fujikuroi* SC	*TEF1/2*	CBS 138572	https://www.fusarium.org/details/23/877	99.85%	97.62%	Ha1	Givat Yoav, Golan Heights
	7cf/11ar	*Fusarium acutatum*, *F. fujikuroi* SC	*RPB2*	CBS 137545	https://www.fusarium.org/details/23/875	99.77%	89.20%		
E6	E1/E2	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 32544	https://www.fusarium.org/details/23/492	99.70%	90.48%	Ha1	Givat Yoav, Golan Heights
	Fa/R8	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*RPB1*	CBS 121450	https://www.fusarium.org/details/23/479	91.48%	44.90%		
E7	E1/E2	*Neocosmospora gamtoosensis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 146502	https://www.fusarium.org/details/23/525	100%	87.29%	Ha1	Givat Yoav, Golan Heights
	7cf/11ar	*Neocosmospora solani*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 117149	https://www.fusarium.org/details/23/735	99.74%	79.45%		
E8	E1/E2	*Neocosmospora gamtoosensis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 146502	https://www.fusarium.org/details/23/525	100%	87.66%	Ha2	Givat Yoav, Golan Heights
	Fa/R8	*Neocosmospora solani*, *Neocosmospora* (previously the *F. solani*) SC	*RPB1*	JW 191039	https://www.fusarium.org/details/23/1337	97.98%	71.44%		
E9	E1/E2	*Neocosmospora gamtoosensis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 146502	https://www.fusarium.org/details/23/525	99.84%	88.27%	Ha2	Givat Yoav, Golan Heights
	7cf/11ar	*Neocosmospora solani*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 43474	https://www.fusarium.org/details/23/749	99.77%	89.68%		

Table 3. *Cont.*

Isolate	Primer	*Fusarium* spp.	Gene	Accession		Similarity	Overlap	Onion Cultivar	Collection Site [c]
E10	E1/E2	*Fusarium acutatum, F. fujikuroi* SC	*TEF1/2*	CBS 137545	https://www.fusarium.org/details/23/875	99.85%	84.29%	Ha1	Givat Yoav, Golan Heights
	7cf/11ar	*Fusarium acutatum, F. fujikuroi* SC	*RPB2*	CBS 137545	https://www.fusarium.org/details/23/875	99.19%	89.41%		
E11	E1/E2	*Fusarium acutatum, F. fujikuroi* SC	*TEF1/2*	CBS 137545	https://www.fusarium.org/details/23/875	99.70%	96.22%	Ha1	Givat Yoav, Golan Heights
	7cf/11ar	*Fusarium acutatum, F. fujikuroi* SC	*RPB2*	CBS 137545	https://www.fusarium.org/details/23/875	99.88%	87.64%		
E13	E1/E2	*Fusarium acutatum, F. fujikuroi* SC	*TEF1/2*	CBS 402.97	https://www.fusarium.org/details/23/31	100%	77.12%	Orlando	Yiron, Galilee
	7cf/11ar	*Fusarium acutatum, F. fujikuroi* SC	*RPB2*	CBS 137545	https://www.fusarium.org/details/23/875	99.18%	89.19%		
E14	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54966	https://www.fusarium.org/details/23/509	99.69%	89.62%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 28563	https://www.fusarium.org/details/23/486	98.09%	73.39%		
E15	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 121450	https://www.fusarium.org/details/23/479	99.70%	93.93%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 43441	https://www.fusarium.org/details/23/501	98.03%	66.10%		
E18	E1/E2	*Fusarium acutatum, F. fujikuroi* SC	*TEF1/2*	CBS 401.97	https://www.fusarium.org/details/23/32	100%	75.88%	Orlando	Yiron, Galilee
	Fa/R8	*Fusarium acutatum, F. fujikuroi* SC	*RPB1*	CBS 137545	https://www.fusarium.org/details/23/875	99.64%	75.57%		
E19	E1/E2	*Fusarium acutatum, F. fujikuroi* SC	*TEF1/2*	CBS 401.97	https://www.fusarium.org/details/23/32	100%	74.57%	Orlando	Yiron, Galilee
	7cf/11ar	*Fusarium acutatum, F. fujikuroi* SC	*RPB2*	CBS 137545	https://www.fusarium.org/details/23/875	99.07%	87.55%		
E20	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	BS 121450	https://www.fusarium.org/details/23/479	99.70%	94.05%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 475.67	https://www.fusarium.org/details/23/478	99.47%	78.73%		
E21	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54983	https://www.fusarium.org/details/23/510	96%	99.42%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 475.67	https://www.fusarium.org/details/23/478	97.53%	79.03%		

Table 3. *Cont.*

Isolate	Primer	*Fusarium* spp.	Gene	Accession		Similarity	Overlap	Onion Cultivar	Collection Site [c]
E22	E1/E2	*Fusarium acutatum, F. fujikuroi* SC	*TEF1/2*	CBS 402.97	https://www.fusarium.org/details/23/31	100%	75.88%	Orlando	Yiron, Galilee
	7cf/11ar	*Fusarium acutatum, F. fujikuroi* SC	*RPB2*	CBS 137545	https://www.fusarium.org/details/23/875	99.07%	89.29%		
E23	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54966	https://www.fusarium.org/details/23/509	99.22%	90.24%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 475.67	https://www.fusarium.org/details/23/478	97.86%	70.14%		
E24	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54983	https://www.fusarium.org/details/23/510	99.53%	90.90%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 475.67	https://www.fusarium.org/details/23/478	98.95%	77.41%		
E26	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54983	https://www.fusarium.org/details/23/510	99.69%	90.51%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 43441	https://www.fusarium.org/details/23/501	99.66%	91.81%		
E27	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54983	https://www.fusarium.org/details/23/510	82% 84%	99.56% 98.98%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora variasi, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 146890	https://www.fusarium.org/details/23/1313	93.20%	51.97%		
E28	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 54983	https://www.fusarium.org/details/23/510	99.68%	89.36%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 54983	https://www.fusarium.org/details/23/510	94.87%	67.59%		
E29	E1/E2	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 121450	https://www.fusarium.org/details/23/479	100%	94.86%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis, Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 28563	https://www.fusarium.org/details/23/486	99.54%	88.88%		

Table 3. *Cont.*

Isolate	Primer	*Fusarium* spp.	Gene	Accession		Similarity	Overlap	Onion Cultivar	Collection Site [c]
E30	E1/E2	*Fusarium fabacearum*, *F. oxysporum* SC	*TEF1/2*	CBS 144742	https://www.fusarium.org/details/23/160	99.67%	74.05%	Orlando	Yiron, Galilee
	7cf/11ar	*Fusarium inflexum*, *F. oxysporum* SC	*RPB2*	CBS 716.74	https://www.fusarium.org/details/23/207	99.77%	89.27%		
E31	E1/E2	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 121450	https://www.fusarium.org/details/23/479	99.55%	80.94%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora ipomoeae*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 833.97	https://www.fusarium.org/details/23/534	97.92%	52.91%		
E32	E1/E2	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	NRRL 43441	https://www.fusarium.org/details/23/501	99.70%	95.01%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	LC13827	https://www.fusarium.org/details/23/2031	95.53%	63.17%		
E33	E1/E2	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 121450	https://www.fusarium.org/details/23/479	99.85%	93.93%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	NRRL 43441	https://www.fusarium.org/details/23/501	95.95%	79.74%		
E34	E1/E2	*Fusarium fabacearum*, *F. oxysporum* SC	*TEF1/2*	CBS 144742	https://www.fusarium.org/details/23/160	99.50%	88.01%	Orlando	Yiron, Galilee
	7cf/11ar	*Fusarium inflexum*, *F. oxysporum* SC	*RPB2*	CBS 716.74	https://www.fusarium.org/details/23/207	99.77%	87.36%		
E35	E1/E2	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*TEF1/2*	CBS 121450	https://www.fusarium.org/details/23/479	99.70%	94.18%	Orlando	Yiron, Galilee
	7cf/11ar	*Neocosmospora falciformis*, *Neocosmospora* (previously the *F. solani*) SC	*RPB2*	CBS 475.67	https://www.fusarium.org/details/23/478	96.22%	75.36%		
E36	E1/E2	*Fusarium brevicatenulatum*, F. fujikuroi SC	*TEF1/2*	CBS 143874	https://www.fusarium.org/details/23/375	89.44%	93.70%	Orlando	Yiron, Galilee
	7cf/11ar	*Fusarium inflexum*, *F. oxysporum* SC	*RPB2*	CBS 716.74	https://www.fusarium.org/details/23/207	100%	89.25%		

[a] Identifying the isolates relies on the highest sequence similarity scores of the studied genomic regions using the Fusarioid-ID database (accessible at www.fusarium.org, accessed on 25 March 2024). PCR confirmed all isolates' identities with E1/E2 primers (targeting the *Fusarium* translation elongation factor-1 alpha gene) or the Fa/R8 or 7cF/11aR primers (targeting the RPB1 or RPB2—the RNA polymerase largest or second-largest subunit, Table 1). Data generated by DNA sequencing were deposited in the NCBI repository. GenBank accession numbers for the nucleotide TEF1/2 sequences are presented in the declarations section, "Data Availability Statement." [b] Isolates E1 and E2 were also identified using the FOC-six3 primers (targeting the *F. oxysporum* f. sp. *cepae* secreted in xylem genes 3, Figure 4). [c] Isolates' colonies were collected from onions originating from Givat Yoav (Golan Heights) and Kibbutz Yiron (Galilee, Hula Valley) fields (see Figure 1). [d] SC—species complex.

3.3. Phylogenetic Relationships between the Fusarium Species

The similarity between the sequences (Supplementary file—Figure S1) showed the conserved and distinct DNA regions for all species. Phylogenetic analysis for the identified species (Figures 6 and 7) demonstrated the relatively close relationships between *F. oxysporum* f. sp. *cepae*, *F. acutatum* species, and the separate branch of *Neocosmospora* isolates. Interestingly, *F. oxysporum* f. sp. *cepae* was found only in the Givat Yoav (Golan Heights) field. At the same time, *F. inflexum* (*F. oxysporum* SC) was isolated only from the Kibbutz Yiron (Galilee, Hula Valley) area. In contrast, *F. acutatum* species were dispersed in both fields. Furthermore, as in the ISSR DNA fingerprinting, it could be seen that all species included sub-branches (clades), which indicates that they undergo further division into subspecies.

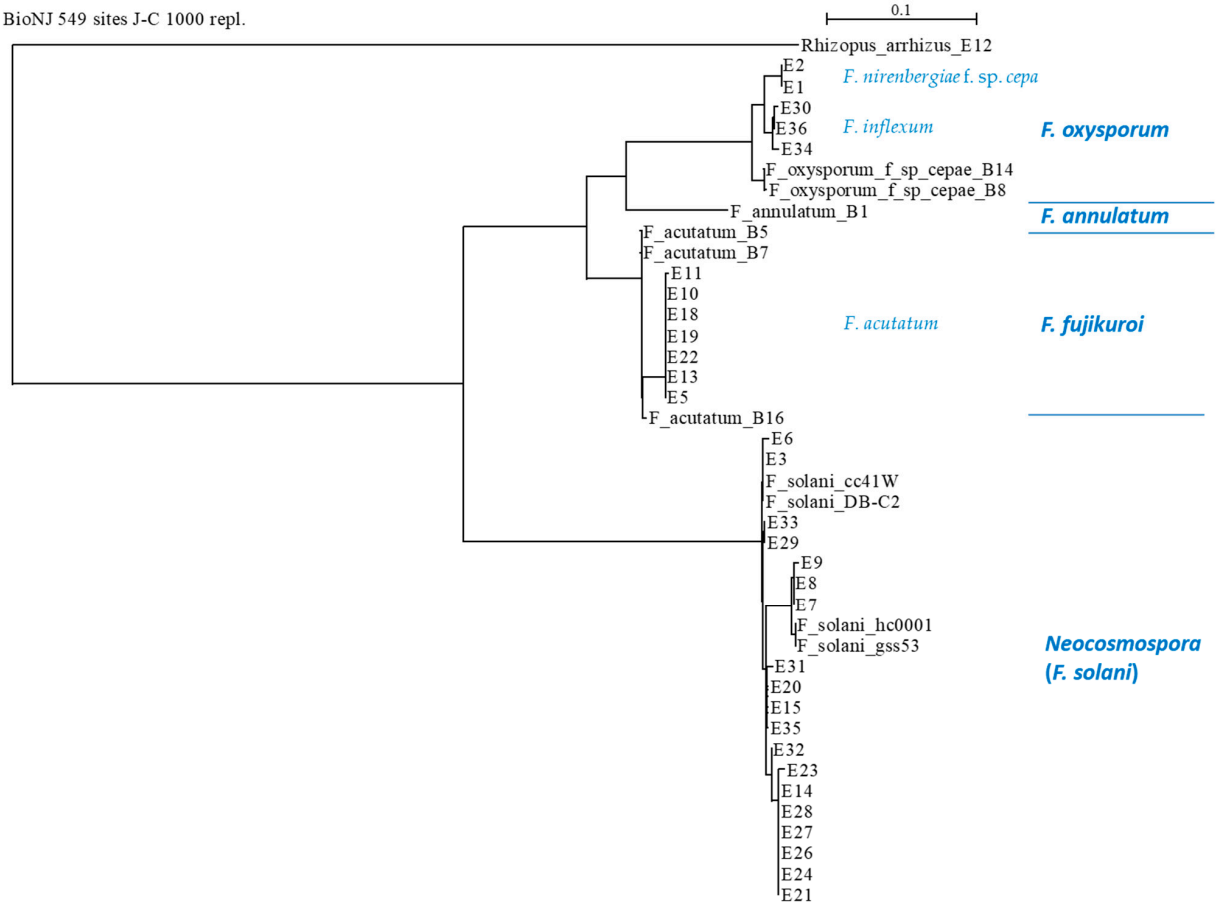

Figure 6. Phylogenetic analysis of the TEF1 gene of the *Fusarium* isolates (presented in Table 3). The upper part's scale describes the genetic resemblance of the isolates. The SeaView version 5.0 program (http://doua.prabi.fr/software/seaview, accessed on 1 April 2024) generated the phylogenetic tree. The alignment was performed using the distance-based method with the default parameters (BioNJ (neighbor-joining algorithm), distance (maximum likelihood), Jukes–Cantor (J-C), bootstrap with 1000 replicates, and excluded positions with gaps). The phylogenetic tree was charted with the forward TEF1-*Fusarium*-specific (E1/E2) primers. The analysis contained the TEF1 gene from the reference strains to assist with the taxonomic assignment. These included previously identified *Fusarium* species in the Golan Heights, Israel (isolates B1, B5, B7, B8, B14, and B16 [21]) and four reference *Neocosmospora* (*F. solani*) species that were taken from the GenBank: isolate cc41W (HQ731052.1), strain hc0001 (KP143718.1), isolate DB-C2 (KY486693.1), and strain gss53 (MH341207.1). Also, an outgroup was set using the onion pathogen *Rhizopus arrhizus* (E12 strain, MK174988.1), isolated and identified as part of the current research.

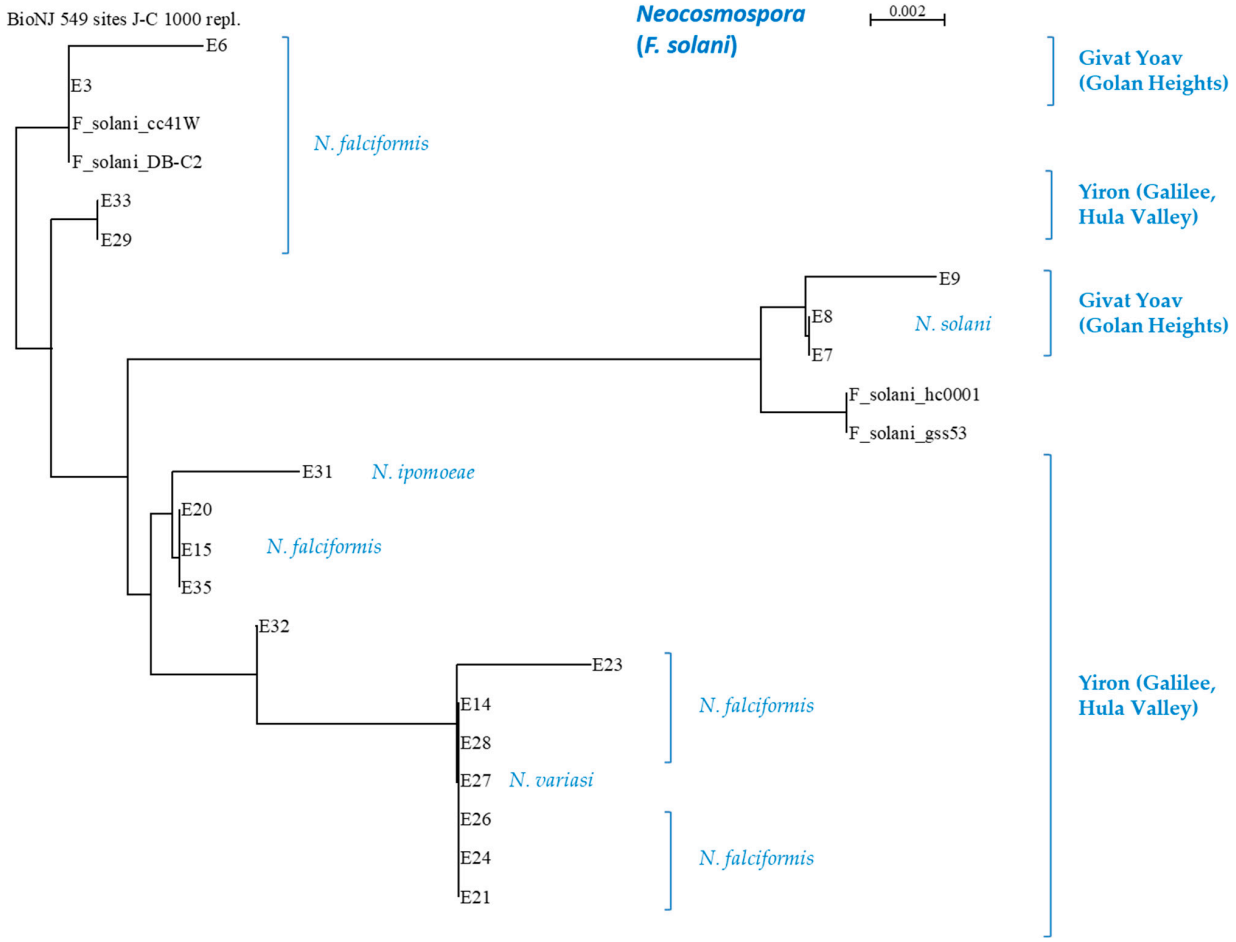

Figure 7. Phylogenetic analysis of the *Neocosmospora* (*F. solani*) species' complex branches. The analysis was outlined in Figure 6, and the *Fusarium* isolates were presented in Table 3.

Such subspecies families can be easily noted in the *Neocosmospora* branch (Figure 7), where the subdivision is related to the collection site and/or the onion cultivar source. An agreement exists between the ISSR and the phylogenetic analysis regarding some of the sub-branches. For example, the E7, E8, and E9 isolates (*N. falciformis*) are differentiated as separate branches of *Neocosmospora* SC in the phylogenetic tree and share a unique DNA profiling in the ISSR results. This compatibility between the two methods also exists with respect to the E14, E21, E23, E24, E26, E27, and E28 isolate groups. Yet, some isolates are associated differently (i.e., belong to different subspecies families) by the two methods (for example, E21, E24, and E26).

3.4. Geographic Distribution, Composition, and Incidence of the Fusarium Species Involved in Onion Basal Rot Disease in Northeastern Israel

The segmentation of isolates according to their place of origin and the host species revealed an interesting pattern (Figure 8). In yellow onions of the Orlando cv. grown in Galilee (Hula Valley), *Neocosmospora* SC was found with the other two species, *F. inflexum* (*F. oxysporum* SC) and *F. acutatum*. In contrast, the Golan Heights bulb sample *Fusarium* spp.'s colonization was divided between species populating red or yellow onion cultivars. The Golan Heights results supported the findings of the work by Kalman et al. [21]. According to the former and current reports, *F. oxysporum* f. sp. *cepae* is abundant in red onions (Ha4/Ha2 cv.), while *F. acutatum* dominates yellow onions (Ha1 cv.). It was curious to discover that *Neocosmospora* SC inhabited both onion cultivars in the Golan Heights.

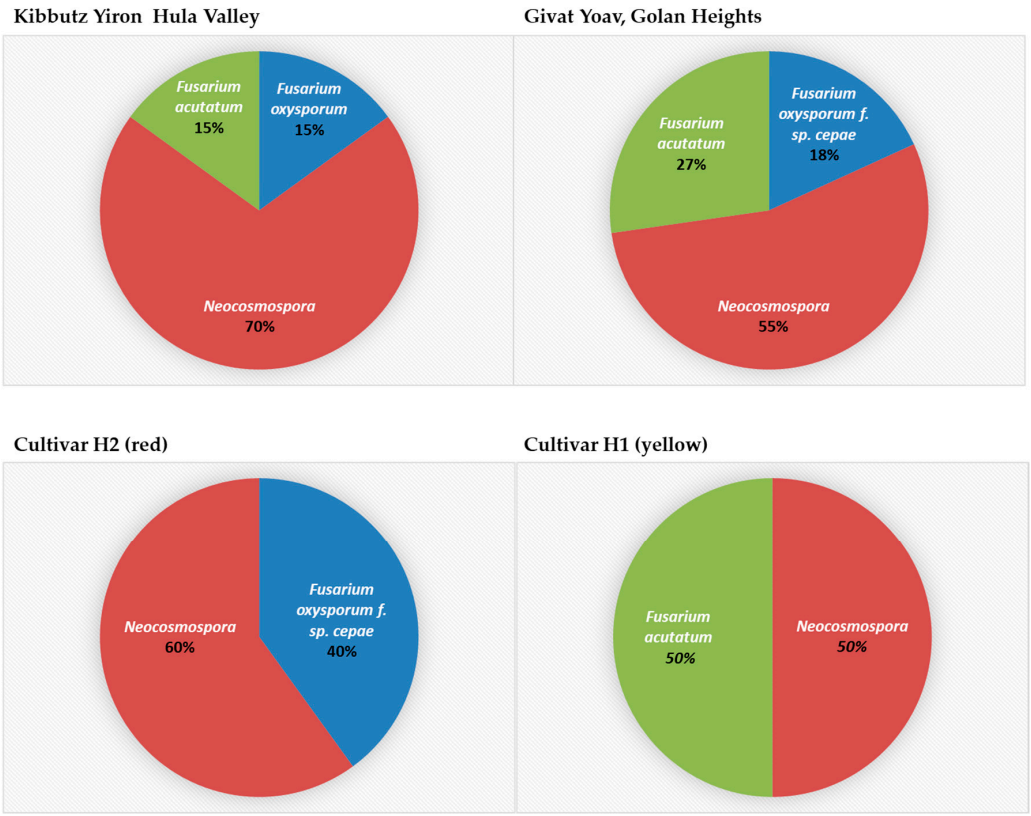

Figure 8. The composition and incidence of *Fusarium* species involved in onion basal rot disease in northeastern Israel. Thirty-one *Fusarium* isolate colonies were collected from onions originating from the Givat Yoav (Golan Heights) and Kibbutz Yiron (Galilee, Hula Valley) fields (see Figure 1). These isolates were grouped according to their collection area (**upper panel**) or onion cultivar source (**lower panel**). Their prevalence (in percentages) is presented.

3.5. Pathogenicity Tests

Fusarium spp. can infect onion plants in various ways, leading to observable symptoms in small seedlings and different plant organs of mature plants. These include the roots, basal stem plate, leaves, and bulb scales [28,29]. Onion seedling and bulb inoculation assays were used to determine the virulence of selected *Neocosmospora* (*F. solani*) isolates. These pathogenicity trials were also aimed at completing Koch's postulates. The onion seedling assay was performed as previously described [21] (Figures 9–11, Supplementary file—Table S2). After nine days of incubation, white mycelia grew on or near the onion seeds as a food source while reducing their developmental rate (Figure 9, see, for example, isolates E3 and E21). The germination percentages were similar (p = 0.17–0.56) in all treatments, ranging between 90 and 100% (Supplementary file—Table S2).

Assessing the sprouts' fresh biomass (Figure 10) and epicotyl emergence number (Figure 11) in each assay plate allows for a more accurate evaluation of the isolates virulence. The *Neocosmospora* isolates E3, E7, and E21 (and, to a lesser extent, E14) caused significant (28–35% reduced biomass, $p < 0.05$) sprouting development repression in the yellow Orlando cv. This growth suppression is expressed in significantly lower shoot emergence percentages in isolates E3, E7, and E9. Isolates E3, E14, and E21 were also the most aggressive toward the red Noam cv. seedlings, though with statistical significance ($p < 0.05$), reached only in the E3 epicotyl emergence evaluation (biomass reduction was 17–21%, p = 0.62–0.90). Compared to the positive control species *F. acutatum* (B5 isolate), the *Neocosmospora* SC isolates were (in most cases) less aggressive in these tests (11–54% higher sprout biomass and improved epicotyl emergence, $p < 0.05$).

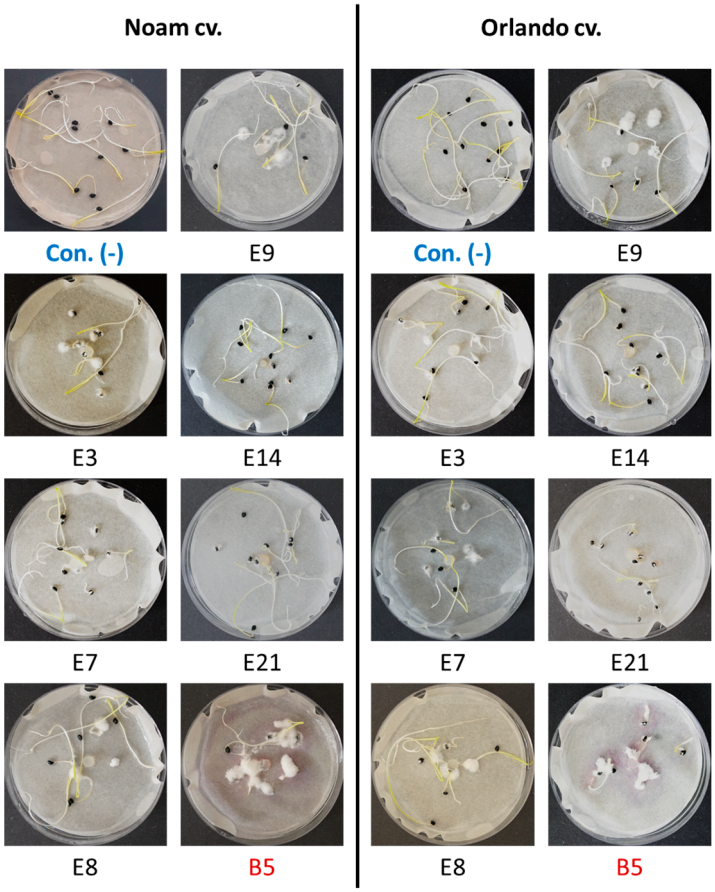

Figure 9. The onion germination pathogenicity assay. Onion seeds from the Orlando (Riverside) and Noam cultivars were used for the pathogenicity test of selected *Neocosmospora* (*F. solani*) isolates (see Table 3). Each group of 10 germinating sprouts was inoculated with a 6 mm diameter disc from a 5-day *Fusarium* sp. colony in the center of a Petri dish. The *F. acutatum* (isolate B5 [21], highlighted in red) was used as a positive control. The control group (Con. [-], highlighted in blue) was grown without inoculation. Photos of representative seeds' plates, taken nine days post incubation at 28 ± 1 °C in the dark, show minor or massive *Fusarium* white mycelial growth on or near the onion seeds, attributed to their growth suppression.

The bulb infection assay results (Figures 12 and 13) were similar to the seedlings' assay outcome. The intact onion bulb virulence assay rapidly assesses the *Fusarium* isolates' ability to invade and thrive in host tissues [21]. In this assay, onion bulbs of the Orlando and Noam varieties were inoculated with six selected *Neocosmospora* isolates, resulting in the appearance of early symptoms two weeks post-infection. The bulbs' basal plate exterior and interior tissue decay (up to 45% and 20% necrotic lesion dimensions and severity) and mycelial growth emergence on the bulb surface all indicated infection. The symptoms observed in this assay closely resembled those surveyed in onion fields naturally infected with the pathogen (Figure 2). Here, also (as in the seedling assay), the *Neocosmospora* isolates were less aggressive (41–45% less necrotic) than *F. acutatum* (B5 isolate). Also, the Orlando cv. was more susceptible to FBR than the Noam cv. Among the *Neocosmospora* SC isolates tested, E3 (*N. falciformis* isolate) was the most virulent strain in the bulbs' pathogenicity assay, with a significantly larger necrotic lesion than the control in the Orlando cv. At the end of the experiment, *Neocosmospora* species were re-isolated from the infected bulbs and identified to satisfy Koch's postulates.

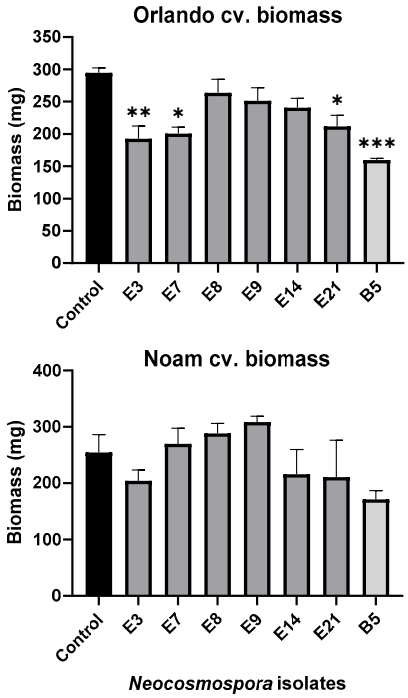

Figure 10. Seedlings' fresh biomass in the pathogenicity experiment (presented in Figure 9). The wet biomass of the resulting seedlings was measured after a nine-day incubation period. The standard error of the mean of four replicates is shown by the vertical upper bars, with asterisks above the error bars indicating a significant difference (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$) between each group and the control in the one-way analysis of variance (ANOVA).

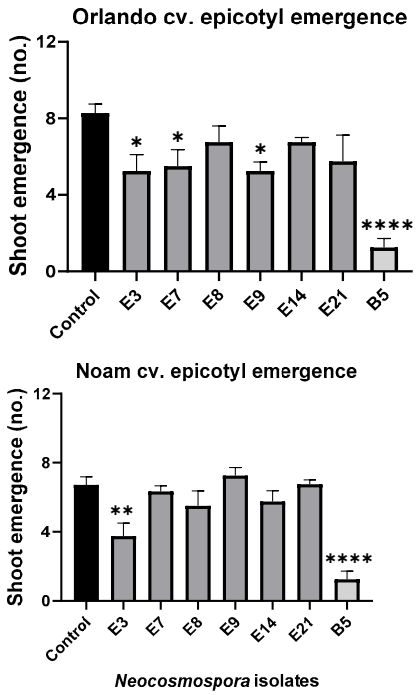

Figure 11. Seedlings' epicotyl emergence number (out of ten per plate) in the pathogenicity experiment (presented in Figure 9). The standard error of the mean of four replicates is shown by the vertical upper bars, with asterisks above the error bars indicating a significant difference (* $p < 0.05$, ** $p < 0.005$, **** $p < 0.00005$) between each group and the control in ANOVA test.

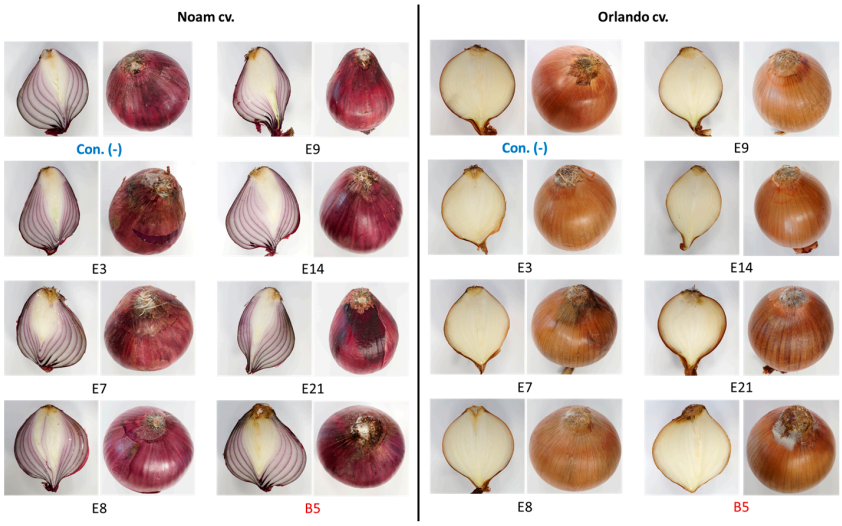

Figure 12. Onion bulb inoculation assay. The assay evaluated the pathogenicity of selected *Neocosmospora* isolates (listed in Table 3). The procedure involved injecting a conidial suspension into the basal plate of the Orlando and Noam cultivar bulbs and then incubating them in moisture bags for two weeks at 28 ± 1 °C in the dark. The *F. acutatum* (isolate B5 [21], highlighted in red) was used as a positive control. The control group (Con. [-], highlighted in blue) was injected with DDW instead of fungal mycelia and spores. The external symptoms on the onion's basal plate and a cross-section of the bulbs were examined, which revealed the development of white hyphae on the outer surface, accompanied by onion tissue decay.

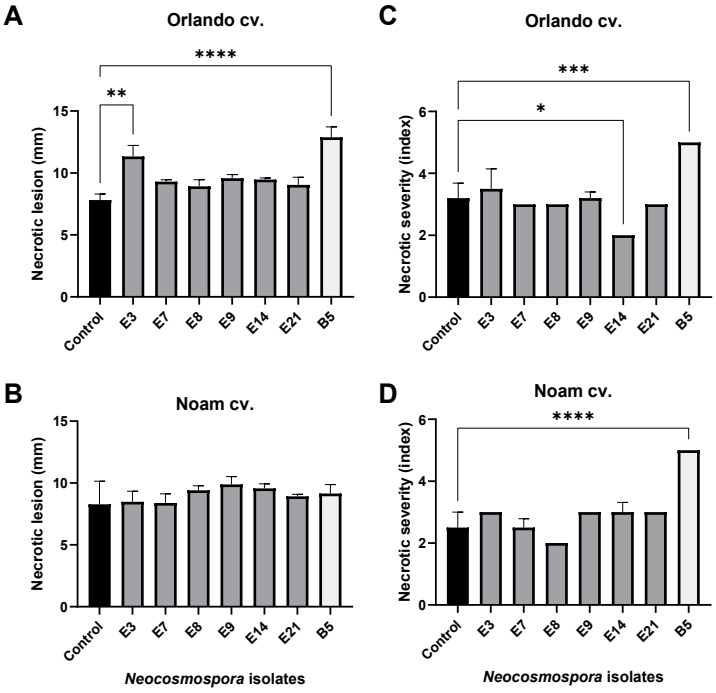

Figure 13. Quantitative assessment of the disease symptoms in the onion bulb inoculation assay (described in Figure 12). Each onion bulb's necrotic lesion dimensions (**A**,**B**) and severity (**C**,**D**) were evaluated. The necrotic lesion dimensions were measured as the length from an onion's lower (root) tip to the scales (fleshy leaves). The necrotic lesion severity was assessed using five categories, of which 5 indicated severe rotting and 1 indicated healthy tissue. The standard error of the mean of 4–5 replications is shown by the vertical upper bars, with asterisks above the error bars indicating a significant difference (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.00005$) between the groups in ANOVA test.

4. Discussion

Fusarium basal rot (FBR) places a significant limitation on *Allium* production worldwide [2]. Damage by the disease can be observed throughout the entire crop cycle. Here, we report a new species complex of the *Fusarium* genus, *Neocosmospora* (previously *F. solani*), which also contributes to the FBR disease epidemic in onions along with *F. oxysporum* SC and *F. acutatum* in the northeastern region of Israel.

This species was already registered as one of the species involved in *Allium* FBR worldwide [2]. Such reports on onion plants include those from Serbia, Sri Lanka, Iran, and Vietnam [3–6]. In addition to uncovering *Neocosmospora* SC as an FBR causal agent in northeastern Israel, the composition and prevalence of *Fusarium* species were analyzed in two commercial fields and three onion cultivars. This study revealed an interesting pattern. *Neocosmospora* SC appeared to be a generalist pathogen group with a weak host specialization and lesser virulence capability than the other more aggressive specialist *Fusarium* species (adapted to a narrow host range). Those included *F. oxysporum* f. sp. *cepae*, dominating red onions (Ha4/Ha2 cv.), and *F. acutatum*, abundant in yellow onions (Ha1 cv.) [21]. This *Fusarium* species composition was characteristic of the Golan Heights field sampled. At the same time, all three *Fusarium* species were found together in the Galilee (Hula Valley) field (planted with a different yellow onion cultivar), about 40 km to the north. Thus, it seems a high host specialization degree is characteristic of some *Fusarium* species, as reported previously [21]. Still, a different pathobiome pattern (i.e., *Fusarium* species composition and incidence) exists depending on the host plant cultivar or location, as demonstrated here.

A previous study in one of the regions studied in this work (the Golan Heights) with the same onion cultivars and the same soil [9] revealed that *F. oxysporum* f. sp. *cepae* exhibited greater aggression towards the red onion Noam cv. At the same time, *F. acutatum* was more virulent towards the yellow Orlando cv. This observation could be attributed to the origin of these pathogens, as *F. oxysporum* f. sp. *cepae* was isolated from the red onion variety (Ha4/Ha2 cv.). In contrast, *F. acutatum* originated from the yellow Orlando cv. ([21] and the current work). In addition, co-inoculation of both pathogens resulted in severe disease in the red Noam cv., similar to the *F. oxysporum* f. sp. *cepae* single infection, but with reduced disease symptoms in the yellow Orlando cv. [9]. This result suggests that antagonistic interactions among certain onion genotypes may exist within the *Fusarium* population.

How does the presence of *Neocosmospora* species affect the onion basal rot disease outbreak? Does it comprise harmful endophyte species or high-virulence disease agents? Does this species complex balance or restrain the more aggressive *Fusarium* species? These are excellent questions to follow up on. The different combinations of *Fusarium* species in diverse onion species result from the host plant and the environment. They raise fascinating questions about the nature of intraspecies relationships in *Fusarium* populations and their interactions with the host plant. For example, are these populations fixed or altered according to plant developmental stage and season-related climatic conditions?

Previous studies provide some clues to the answers to these questions. For instance, it was observed that discernible divergences in pathogenicity across and within *Fusarium* species resulted in contrasting disease pathogenesis outcomes [2,30,31]. Such variability appears to be more related to the host plant than the geographical origin or climatic variables, which have a lesser impact in some instances [32]. The mechanism behind the FBR pathogenesis is now gradually being revealed. The disease severity results from pathogen metabolites and virulence factors interacting with the plant defense system. One such metabolite is fumonisin B1, secreted by *F. proliferatum* [1]. This toxin's expression can vary depending on the infected host organ and phenological development stage. In response, the plant's defense-related genes are expressed differentially during the seedling and bulb infection. These plant metabolites include lipoxygenase (*LOX2*), phenylalanine ammonia-lyase (*PAL1*, *PAL2*), anthocyanidin synthase (*ANS*), chalcone synthase (*CHS*), and pectin methyl esterase (*PME*) [1]. Thus, the plant's defense variations are primarily linked

to pathogen specialization towards specific host species, modulated by genetic mechanisms that repress host defense responses.

The current study's findings are economically significant since each *Fusarium* species may react differently to control treatments [9]. Thus, knowing the exact population structure may assist in tailoring FBR protection to maximize its efficiency. The strategies currently employed in Israel to manage FBR disease are limited and consist of a four-year crop rotation cycle and soil disinfection using metam sodium [21]. However, despite these measures, the disease persists and is spreading to new areas where contaminated equipment and agricultural tools, such as harrows and plows, and the workforce unintentionally contribute to its propagation [21]. The problem is not unique to Israel but crosses borders. According to a review by Le et al. [2], *Allium* producers worldwide continue to face a significant disease problem despite implementing numerous control measures.

A recent study [9] explored the potential of chemical control methods in mitigating FBR disease damage in Israel. Initially, novel substances effective against the pathogens involved were identified using a plate screening technique. Subsequently, selected formulations from earlier trials in seedlings were evaluated for an entire growing season. One of the preparations, prochloraz, added to the irrigation, displayed efficacy against the principal causal agent of onion FBR disease, *F. oxysporum* f. sp. *cepae* (B14 isolate). However, it was relatively less effective against *F. acutatum*. Another compound based on fludioxonil + sedaxen (Fl-Se) applied in a seed coating could protect both onion cultivars against the two *Fusarium* species tested. Thus, a combined treatment that relies on both prochloraz and Fl-Se could be preferable.

Other important conclusions could be drawn from the results presented here. For instance, it was shown for the first time that the level of contamination in some fields is significantly higher than previously assumed and reaches 8% in certain varieties. While such a high incidence is alarming, it may be affected by the level of inbreeding. Since onion is an outcrossing species, it may suffer inbreeding depression [33]. The different lines used in onion hybrid cultivar development are often inbred to a certain extent to ensure uniformity within the hybrid cultivar. Inbred lines are frequently weaker in their growth than hybrid or open-pollinated cultivars. Thus, the survey presented here must be followed by a more comprehensive and dedicated study to evaluate the disease severity in different commercial onion cultivars in various geographic regions, considering the pollination method and other cultivation aspects.

Moreover, field losses are only a partial picture of the disease impact. The disease spread in onions is enhanced during storage, particularly in open sheds or packing houses. In this scenario, the disease can spread to other onions, and there is also a concern that infected bulbs that do not show visible symptoms could make their way to markets throughout the country. This concern increases significantly in light of the presence of toxins known to be produced by these pathogens [34].

According to Cramer [28], losses resulting from FBR can vary depending on growth stages and regions. Among the damping-off pathogens, *Fusarium* spp. can cause up to 70% of damage in nurseries [35]. *Fusarium* spp. can also lead to significant losses in bulbs, with reported losses of up to 50% in the field and 30–40% in storage in Asia [35,36]. Dauda and colleagues [8] observed FBR affecting 50% of seedlings in African growth areas. In southern New Mexico (USA), Cramer [28] reported a 40% and 29% disease incidence for fall-planted and spring-planted cultivars, respectively. Meanwhile, in Zambia, even when cultivated in virgin soil, the FBR prevalence was found to be high, with 80–90% of transplants infected by *F. oxysporum* f. sp. *cepae*, leading to significant losses in post-transplanting seedlings (44%) and potential yield (69%) [37]. Organic farms typically experience higher losses than conventional farms [38].

So, what future directions are needed to create efficient strategies for managing FBR? One (so far poorly explored) option is integrated pest management [2]. Integrating disease-resistant crops and biological control measures can provide proactive prevention against

FBR disease damage. This approach should also involve using disease-free planting materials and regularly maintaining field hygiene to limit the spread of infections.

5. Conclusions

Fusarium basal rot disease (FBR) in onion (*Allium cepa*) is common worldwide, causing severe damage typified by an infection that spreads from the roots to the onion stem base and leaves. Significant knowledge gaps exist today regarding Israel's FBR, the pathogen population involved, and the damage they cause. The current study analyzed the composition and prevalence of *Fusarium* species in two commercial fields in northeastern Israel, one in the Golan Heights and the other in Galilee (Hula Valley). The results revealed for the first time that *Neocosmospora* (previously *F. solani*) SC is part of the Fusarium population in onion FBR in northeastern Israel, and we found it to be the most common Fusarium in bulbs sampled from both areas. Furthermore, while in yellow onions of the Orlando cv. grown in the Galilee field, this species was found with two other species, *F. oxysporum* f. sp. *cepae* and *F. acutatum*, the Golan Heights field's composition of *Fusarium* species was divided between onion cultivars. The red Ha2 cv. onions were populated by *F. oxysporum* f. sp. *cepae,* while the yellow Ha1 cv. onions were infected by *F. acutatum*. Meanwhile, *Neocosmospora* SC was found in both onion varieties. An in vitro seed and bulb pathogenicity assay showed that *Neocosmospora* species are moderately aggressive disease agents. Yet, the impact of these species' combinations and interspecies relationships on host plant health is yet to be explored. The results of this and other global studies indicate that the Fusarium pathobiome composition and structure require specifically adapted pest control solutions since each *Fusarium* species may react differently with fungicide treatments.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10040373/s1, Figure S1: Sequence alignment of the TEF1 gene of the *Fusarium* isolates (presented in Table 3); Figure S2: Full-length gels for the DNA fingerprinting of the *Fusarium* isolates shown in Figure 7A,B; Table S1: NCBI BLASTN identification of the Fusarium isolates from this study; Table S2: Seed germination in the seedling pathogenicity assay for *Neocosmospora* (*F. solani*) isolates.

Author Contributions: Conceptualization, O.D., E.D. and E.M.; data curation, O.D. and E.D.; formal analysis, O.D. and E.D.; funding acquisition, O.D. and E.M.; investigation, O.D. and E.D.; methodology, O.D. and E.D.; project administration, O.D.; resources, O.D. and E.M.; supervision, O.D.; validation, O.D. and E.D.; visualization, O.D. and E.D.; writing (original draft), O.D.; writing (review and editing), O.D., E.D. and E.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data generated by DNA sequencing were deposited in the NCBI repository. GenBank accession numbers for the nucleotide sequences are in Appendix A. All other data generated or analyzed during this study are included in this published article (and its Supplementary Information Files).

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Conflicts of Interest: The authors declare no conflicts of interest. All authors have read and agreed to the published version of the manuscript.

Appendix A. GenBank Accession Numbers for the Nucleotide Sequences (https://www.ncbi.nlm.nih.gov/, Accessed on 1 April 2024)

Isolate	TEF1 Gene		RPB2 Gene		RPB1 Gene	
E1	OR206059	https://www.ncbi.nlm.nih.gov/nuccore/OR206059	PP429247	https://www.ncbi.nlm.nih.gov/nuccore/PP429247		
E2	OR206058	https://www.ncbi.nlm.nih.gov/nuccore/OR206058	PP429246	https://www.ncbi.nlm.nih.gov/nuccore/PP429246.1/		
E3	OR206086	https://www.ncbi.nlm.nih.gov/nuccore/OR206086	PP429275	https://www.ncbi.nlm.nih.gov/nuccore/PP429275		
E5	OR206068	https://www.ncbi.nlm.nih.gov/nuccore/OR206068	PP429257	https://www.ncbi.nlm.nih.gov/nuccore/PP429257		
E6	OR206085	https://www.ncbi.nlm.nih.gov/nuccore/OR206085			PP429274	https://www.ncbi.nlm.nih.gov/nuccore/PP429274
E7	OR206084	https://www.ncbi.nlm.nih.gov/nuccore/OR206084	PP429273	https://www.ncbi.nlm.nih.gov/nuccore/PP429273		
E8	OR206083	https://www.ncbi.nlm.nih.gov/nuccore/OR206083			PP429272	https://www.ncbi.nlm.nih.gov/nuccore/PP429272
E9	OR206082	https://www.ncbi.nlm.nih.gov/nuccore/OR206082	PP429271	https://www.ncbi.nlm.nih.gov/nuccore/2693348682		
E10	OR206064	https://www.ncbi.nlm.nih.gov/nuccore/OR206064	PP429252	https://www.ncbi.nlm.nih.gov/nuccore/PP429252		
E11	OR206063	https://www.ncbi.nlm.nih.gov/nuccore/OR206063	PP429251	https://www.ncbi.nlm.nih.gov/nuccore/PP429251		
E12	OR206088	https://www.ncbi.nlm.nih.gov/nuccore/OR206088				
E13	OR206067	https://www.ncbi.nlm.nih.gov/nuccore/OR206067	PP429256	https://www.ncbi.nlm.nih.gov/nuccore/PP429256		
E14	OR206081	https://www.ncbi.nlm.nih.gov/nuccore/OR206081	PP429270	https://www.ncbi.nlm.nih.gov/nuccore/PP429270		
E15	OR206080	https://www.ncbi.nlm.nih.gov/nuccore/OR206080	PP429269	https://www.ncbi.nlm.nih.gov/nuccore/PP429269		
E18	OR206065	https://www.ncbi.nlm.nih.gov/nuccore/OR206065			PP429253	https://www.ncbi.nlm.nih.gov/nuccore/PP429253
E19			PP429254	https://www.ncbi.nlm.nih.gov/nuccore/PP429254		
E20	OR206079	https://www.ncbi.nlm.nih.gov/nuccore/OR206079	PP429268	https://www.ncbi.nlm.nih.gov/nuccore/PP429268		
E21	OR206078	https://www.ncbi.nlm.nih.gov/nuccore/OR206078	PP429267	https://www.ncbi.nlm.nih.gov/nuccore/PP429267		
E22	OR206066	https://www.ncbi.nlm.nih.gov/nuccore/OR206066	PP429255	https://www.ncbi.nlm.nih.gov/nuccore/PP429255		
E23	OR206077	https://www.ncbi.nlm.nih.gov/nuccore/OR206077	PP429266	https://www.ncbi.nlm.nih.gov/nuccore/PP429266		
E24	OR206076	https://www.ncbi.nlm.nih.gov/nuccore/OR206076	PP429265	https://www.ncbi.nlm.nih.gov/nuccore/PP429265		
E26	OR206075	https://www.ncbi.nlm.nih.gov/nuccore/OR206075	PP429264	https://ncbi.nlm.nih.gov/nuccore/PP429264		
E27	OR206074	https://www.ncbi.nlm.nih.gov/nuccore/OR206074	PP429263	https://www.ncbi.nlm.nih.gov/nuccore/PP429263		
E28	OR206073	https://www.ncbi.nlm.nih.gov/nuccore/OR206073	PP429262	https://www.ncbi.nlm.nih.gov/nuccore/PP429262		
E29	OR206072	https://www.ncbi.nlm.nih.gov/nuccore/OR206072	PP429261	https://www.ncbi.nlm.nih.gov/nuccore/PP429261		
E30	OR206060	https://www.ncbi.nlm.nih.gov/nuccore/OR206060	PP429248	https://www.ncbi.nlm.nih.gov/nuccore/PP429248		
E31	OR206071	https://www.ncbi.nlm.nih.gov/nuccore/OR206071	PP429260	https://www.ncbi.nlm.nih.gov/nuccore/PP429260		

Isolate	TEF1 Gene		RPB2 Gene		RPB1 Gene
E32	OR206070	https://www.ncbi.nlm.nih.gov/nuccore/OR206070	PP429259	https://www.ncbi.nlm.nih.gov/nuccore/PP429259	
E33	OR206069	https://www.ncbi.nlm.nih.gov/nuccore/OR206069	PP429258	https://www.ncbi.nlm.nih.gov/nuccore/PP429258	
E34	OR206062	https://www.ncbi.nlm.nih.gov/nuccore/OR206062	PP429250	https://www.ncbi.nlm.nih.gov/nuccore/PP429250	
E35	OR206087	https://www.ncbi.nlm.nih.gov/nuccore/OR206087	PP429276	https://www.ncbi.nlm.nih.gov/nuccore/PP429276	
E36	OR206061	https://www.ncbi.nlm.nih.gov/nuccore/OR206061	PP429249	https://www.ncbi.nlm.nih.gov/nuccore/PP429249	
B1	OR206094	https://www.ncbi.nlm.nih.gov/nuccore/OR206094			
B5	OR206092	https://www.ncbi.nlm.nih.gov/nuccore/OR206092			
B7	OR206090	https://www.ncbi.nlm.nih.gov/nuccore/OR206090			
B8	OR206091	https://www.ncbi.nlm.nih.gov/nuccore/OR206091			
B14	OR206089	https://www.ncbi.nlm.nih.gov/nuccore/OR206089			
B16	OR206093	https://www.ncbi.nlm.nih.gov/nuccore/OR206093			

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