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Development of New Cotton Varieties Resistant to *Verticillium* and *Fusarium* Wilt

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ABSTRACT

Cotton production worldwide is severely threatened by soil-borne fungal pathogens, primarily *Verticillium dahliae* (VD) and *Fusarium oxysporum* f. sp. *vasinfectum* (FOV). These wilts can cause seedling loss and substantial yield reduction, making the development of resilient cultivars essential. This study utilized an integrated breeding approach, combining marker-assisted selection (MAS) with rigorous phytopathological and biochemical analyses, to accelerate the development of wilt-resistant *Gossypium hirsutum* lines. We screened 10 fungal isolates and 8 complex interlinear hybrids across multiple generations (F₇–F₁₁). Genotyping using six SSR markers, including BNL4003, CM209, and TMB0161, identified the hybrid CBRI_3 as the most promising source of resistance alleles. Biochemical studies confirmed that two hybrids, CBRI_3 and CBRI_8, exhibited superior resistance. In these resistant lines, peroxidase enzyme activity was significantly 1.3–1.6 times higher than that in susceptible controls, promoting vital cell wall reinforcement and lignification, which are key defenses against fungal penetration. This integrated approach successfully led to the isolation of the highly resistant line, L-1077 (derived from CBRI_3). This line maintains high fiber quality (Type IV standard) and superior yield while showing strong resistance to both *V. dahliae* and FOV. The line L-1077 was submitted for patent registration under the variety designation C-6602 (NAP 2020 0007).

1 | Introduction

Cotton (*Gossypium hirsutum* L.) is a globally vital and profitable agricultural commodity cultivated in more than 80 countries. Historically, the practice of cotton cultivation in Central Asia has been traced back to the 6th century BC [1]. Despite its economic importance, global cotton production is severely constrained by devastating soil-borne diseases,

primarily those caused by species within the fungal genera *Fusarium* and *Verticillium* [2–4].

The genus *Fusarium* constitutes a vast, biologically heterogeneous complex of fungi, including over 100 species that function as saprotrophs, facultative parasites, and obligate parasites. This broad host range contributes to disease susceptibility in nearly all crops [5]. The most common fungal diseases are associated with

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species such as *F. solani*, *F. equiseti*, *F. culmorum*, *F. oxysporum*, and *F. chlamydosporum* [6]. While traditional identification relied on morphological traits, such as the typically sickle-shaped macroconidia [7], modern species delineation now utilizes advanced molecular tools, including DNA sequencing of the ITS and EF-1 α regions [8–10].

Previous studies have consistently demonstrated that *Fusarium* species pose a greater threat to cotton seedlings than to plants in later developmental stages. In the United States, particularly in the southeastern states, *Fusarium* wilt (FW), caused by *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) poses a significant challenge to agriculture.

Regional yield losses in susceptible cultivars can range widely, from 10% to 50% [3]. Specifically, in Alabama, losses have been quantified at up to 6%, which is approximately \$10,282,200 annually [11]. In Missouri, seedling diseases have caused greater estimated yield losses over the last two decades than any other cotton ailment. Furthermore, *Fusarium* spp. were detected in over 50% of the cotton seedlings collected annually from all surveyed fields [12].

Fusarium species are among the most widespread and harmful pathogens contributing to seedling mortality in cotton-producing regions globally [13]. This prevalence has been documented in locations including Pakistan, Brazil [14], and throughout the United States, including Arkansas, Louisiana, Georgia, Minnesota, and North Carolina [15], and in extensive areas across the United States (e.g., Texas and Mississippi) [16, 17]. In Upper Egypt, root rot remains a highly detrimental disease, with pathocomplex studies in the Assiut region identifying *F. solani*, *F. moniliforme*, and *Fusarium oxysporum* [18]. Similar sprout damage due to *Fusarium* has also been reported in Australia [19, 20]. Seedling damage caused by *Fusarium* has also been reported in Nigeria [21], with yield losses in Northern India ranging from 5% to 10% [22] and exceeding 20% in parts of Europe and the United States [3, 23].

The pathogenic influence of *Fusarium* spp. on cotton during the vegetative phase results in significant raw cotton harvest losses in various regions of Uzbekistan [24]. An alarming phytosanitary situation exists in areas such as the Bukhara region due to *Fusarium* management challenges. In affected fields, seedling loss can reach 40%–50%, and preharvest loss rates have been reported to be as high as 71% [25]. Furthermore, monitoring studies in southern regions (Surkhandarya and Kashkadarya) during 2015–2016 confirmed the identification of *Fusarium* diseases affecting cotton seedlings [24].

Cotton seedling damage caused by both *Fusarium* and *Verticillium* remains a critical issue in modern cotton agriculture [3, 13, 26]. Specifically, highly pathogenic races of FOV (races 4 and 7) induce severe root rot and vascular wilt, leading to stunted and premature mortality [27]. These aggressive races pose a persistent threat because of their capacity for long-term survival in the soil [28].

Verticillium wilt (VW), caused by *Verticillium dahliae* (VD), severely affects cotton by reducing fiber quality and causing significant yield losses. Documented yield losses from defoliating VD isolates have been substantial, reaching up to 79% in plots with high pathogen densities [29]. VW is prevalent in the western United States, Mediterranean countries, and parts of Asia [30].

The reliance on chemical protection methods is problematic because pesticides are environmentally detrimental and promote pathogen resistance [31]. Consequently, the development of genetically resistant varieties remains the most effective long-term solution. However, conventional breeding methods are slow and often fail to keep pace with pathogen adaptive evolution. This reality mandates that protective strategies begin with the early diagnosis and detection of pathogens in soil and plant tissues.

To accelerate the development of novel disease-resistant cultivars, it is essential to leverage genetic diversity in cotton. Employing DNA markers allows for the rapid incorporation of resistance traits against *Fusarium* and VD. Molecular markers, particularly simple sequence repeats (SSRs), are recognized as effective and reliable tools for genetic diversity analysis, genotyping, and mapping genes of interest, specifically aiding approaches such as marker-assisted selection (MAS) [32, 33].

The primary objective of this study was to accelerate the breeding of cotton cultivars with enhanced resistance to FW and VW by leveraging the power of MAS. We detail an integrative workflow that combines molecular genetic techniques, utilizing established SSR markers linked to resistance Quantitative Trait Loci (QTLs), with conventional breeding methods, including rigorous phytopathological assessments and relevant biochemical analyses. This comprehensive approach, which combines phenotypic selection with genotypic screening, is designed to significantly reduce the timeline required for the successful development and release of new, commercially viable, disease-resistant cotton cultivars.

2 | Materials and Methods

2.1 | Plant Materials

Hybrids obtained from crosses between lines, varieties, and complex crosses between them were used as breeding materials. Sowing of cotton breeding material, evaluation of economically valuable traits, and yield data collection were conducted according to standard procedures in cotton breeding and seed production. Specifically, field plot techniques and experimental design followed Dospekhov [34], while breeding and selection methods were based on Simongulyan et al. [35] and the guidelines outlined in the cotton breeding program of the All-Union Research Institute of Cotton Breeding and Seed Production named after G.S. Zaitsev [36]. This program details the procedures for all stages of breeding, from the initial collection nursery to competitive variety trials.

The following table provides a brief description of the original line forms and varieties used in this study (Table 1).

2.2 | Fungal Materials

The isolates were obtained from a collection of phytopathogens at the Institute of Genetics and Experimental Plant Biology (IGPEB), Tashkent, Uzbekistan. They were originally isolated from soil and diseased cotton plants in different regions of Uzbekistan. Monosporous isolates of *Fusarium* Lk. VD isolated from the collected plants was used to develop an infection background (Table 2).

TABLE 1 | Characterization of cotton accessions by economically valuable traits.

No. Varieties and lines		Origins	Vegetation period, days	Weight of raw cotton of one box (g)	Fiber output (%)	Staple fiber length (mm)	Resistance of plants to <i>V. dahliae</i> under heavily infected field conditions, scores (0 - no disease, 5 - severe disease).
1	C-6524 (variety indicator)	([159-F × 05152] × 159-F)	120	6.12	33.47	34.2	4.26
2	Buxara-6 (variety indicator)	137-F × S-1470	118	6.59	36.38	32.3	4.28
3	L-101	F ₇ S-6530 × Akkurgan-2	118	6.45	38.83	33.6	1.24
4	L-103	F ₆ Omad × Namangan-77	121	5.96	38.78	34.2	1.10
5	L-104	F ₈ Istiqlol × S-6530	113	6.39	39.62	33.3	1.51
6	L-105	F ₇ S-5621 × Namangan-77	122	6.30	39.46	33.6	1.47
7	L-106	F ₆ S-2609 × Omad	117	6.47	37.53	32.6	1.65
8	L-107	F ₇ S-6532 × S-4910	116	6.56	38.72	33.6	1.67
9	F ₆ (L-101 × L-108)		117	5.43	36.17	34.1	1.41
10	F ₆ (L-105 × L-106)		116	6.20	38.24	34.6	1.33
11	F ₆ (L-103 × L-106)		114	5.97	39.16	33.8	0.84
12	F ₆ (L-105 × L-108)		119	6.44	38.00	35.1	0.93
13	F ₆ (L-104 × L-108)		116	6.02	39.17	34.9	1.10
15	Jarkurgan	F ₁₀ ([F ₁ <i>G. thurberi</i> Tod. × <i>G. raimondii</i> Ulbr.] × LSG-187)	127	6.4	36.4	32.8	2.39
16	Namangan-102	159-F × 02654	119	5.9	37.9	33.5	2.81
17	Namangan-34	S-1973 × 02654	120	6.11	39.1	34.4	2.70

Note: The hybrids used in this study were coded for ease of understanding.

TABLE 2 | Isolates from the IGPEB collection of phytopathogens.

Isolate	The area from which the pathogen was introduced	The name of the pathogen
319	Namangan region	<i>F. solani</i>
328	Bukhara region	FOV race 4
379	CBSARI	FOV race 3
404	Bukhara region	<i>F. sporotrichioides</i>
422	—	<i>F. solani</i>
473	—	<i>F. solani</i>
502	Tashkent region	<i>F. proliferatum</i>
509	Tashkent region	<i>F. proliferatum</i>
519	Bukhara region	<i>F. solani</i>
520	Bukhara region	<i>F. solani</i>

2.3 | Isolation of Pure Fungal Cultures From the Soil

The method described by Bilay et al. [37] for isolating pure fungal cultures from soil for laboratory research includes several key steps:

- Soil Preparation: Soil samples were collected from the top 0–10 cm, 10–20 cm, and 20–30 cm layers, ensuring the removal of plant residues, stones, and sand. The soil was then ground and sieved.
- Suspension Preparation: One gram of the prepared soil was added to 99 mL of sterile water, and the mixture was thoroughly shaken for 3 min. The resulting suspension was further diluted in sterile test tubes, repeating the dilution steps until a final concentration of 1:1,000,000 was achieved.
- Inoculation on Culture Media: One milliliter of the prepared suspension was placed in three sterile Petri dishes, to which molten culture media (e.g., DPA or Chapeka agar) was added at 40°C–45°C. The medium was mixed with the suspension, and the Petri dishes were incubated in a thermostat at 25°C for 3 days.
- Colony Growth Assessment: After incubation, the colonies of microorganisms that grew on the medium were counted. The average number of colonies from all dishes was multiplied by the dilution factor to determine the number of microorganisms per gram of soil. These counts were used to estimate the total fungal density. Comprehensive pathogenicity testing was performed on the specific collector strains listed in Table 2, but not on all colonies isolated from the soil.
- Microscopic Examination: For further analysis, microscopic preparations were made from the colonies grown on Petri dishes to examine the morphological features of the fungi.

2.4 | Fungal Isolation and Media Preparation

Initial mycological analysis of the field and soil samples was performed using the standardized procedures established by Khasanov and Glukhova [38]. To characterize the macro-morphology of *Fusarium* Link and VD isolates, the fungi were cultured on potato-glucose/dextrose agar (PDA) supplemented

with 0.3 g/L streptomycin sulfate and 600 mg/L chloramphenicol to ensure purity. For micromorphological analysis, isolates were transferred to Nirenberg medium (synthetic nutrient agar, SNA) [39]. Furthermore, the specific color profiles of the pigments secreted by the isolates were studied on media augmented with 1.0 g/L asparagine, as characterized according to the Bondartsev [40] scale. To ensure that the macroconidia collected for inoculation were uniform in both size and shape, we utilized Leaf-piece Agar (GLA) [41]. Cotton was infected with *Fusarium* pathogens under laboratory conditions, following the method described by Kim et al. [42]. In this experiment, the cotton varieties and hybrids specified in Table 3 were utilized, and the pathogens listed in Table 3 were used for the inoculation procedure. Cotton seeds from each sample (10 plants per sample) were sown in triplicate randomized replicates in cups filled with specially prepared soil for germination. After the emergence of true leaves, the roots were immersed in a conidial suspension (1×10^6 conidia/mL) containing the pathogen for 3 min. The control plants were immersed in distilled water for the same duration. Each infected plant was placed separately in a cup filled with soil.

The resistance of cotton accessions to FW was assessed under both controlled laboratory and field conditions by evaluating visible foliar (leaf) symptoms.

For laboratory assessments, the experiment involved a total of 30 plants for each testing sample, which were sown as 10 plants in three random replicates. After inoculation with a pathogen spore suspension, disease development was monitored for 10-day period. Evaluations were conducted on plots with a natural infection background. Plant survival in each plot was recorded 15 days prior to harvesting. To estimate disease severity, five plants were randomly chosen from each plot as subsamples for analysis. For both trials, disease severity was evaluated based on phenotypic manifestations using the following scale: 0—no changes; 1—slight color change (small spots visible in the longitudinal section of the stem); 2—extended color change, covering one-quarter to one-half of the stem (lighter color); 3—moderate color change, covering more than half of the stem; 4—noticeable darkening of the stem, more intense than in categories 1 and 2; 5—severe damage, with darkening of the vascular system throughout the entire stem.

TABLE 3 | Coding of the breeding materials involved in the experiment.

No.	Varieties and lines	Coded names
1	C-6524 (ind)	C-6524 (ind)
2	Buxara-6 (ind)	Buxara-6 (ind)
3	F ₁₁ (F ₆ [L-106 × L-105] × L-106)	CBRI_1
4	F ₁₁ (F ₆ [L-101 × L-106] × L-105)	CBRI_2
5	F ₁₁ (F ₆ [L-101 × L-105] × L-106),	CBRI_3
6	F ₁₁ (F ₆ [L-105 × L-106] × L-105),	CBRI_4
7	F ₁₁ (F ₆ [L-101 × L-108] × L-103),	CBRI_5
8	F ₁₁ (F ₆ [L-105 × L-106] × L-104),	CBRI_6
9	F ₇ (L-175/276 × Namangan-102),	CBRI_7
10	F ₇ (Jarkurgon × Namangan-34).	CBRI_8

2.5 | PCR Analysis and Genotyping

Genomic DNA was isolated from frozen cotton leaves using the CTAB method with minor modifications [12]. The SSR markers used in this study were selected based on their association with resistance to FW and VW.

Three SSR primers previously identified in association mapping studies as linked to FOV and VD resistance were used for PCR amplification: BNL4003 (145 bp), CM209 (140 bp), and TMB0161 (192 bp) [43]. In the association mapping study by Abdullayev [43], BNL4003 showed a significant correlation ($p \leq 0.05$) with wilt resistance in multiple environments. CM209 exhibited a strong linkage ($p \leq 0.01$) to VD resistance alleles, whereas TMB0161 was significantly associated ($p \leq 0.01$) with fiber length and indirectly with FOV resistance.

PCRs were performed in 10- μ L volumes, each containing 20 ng of cotton template DNA, 10 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂; 50 mM KCl), 0.1 mg/mL BSA, 0.25 mM each dNTP, 0.2 μ M forward and reverse primer mixtures, and 0.25 μ L Taq polymerase. The PCR program consisted of an initial denaturation of DNA at 95°C for 3 min, followed by 45 cycles of 94°C for 30 s, X °C (specific to each primer set) for 30 s, and 72°C for 90 s, with a final extension at 72°C for 5 min.

Polymorphisms in the amplified microsatellite loci were detected by fractionating the PCR products on a 3.5% high-resolution HiRes Agarose gel (GeneMate, Delhi, India).

2.6 | Biochemistry Methods

The interaction between a pathogen and its host typically triggers distinct changes in plant cellular metabolism, most notably in the modulation of the phytoimmunity enzyme peroxidase (PO). A soil-isolated VD strain was used to infect the cotton plants. VD infection in cotton occurs primarily through the root system, which is the primary barrier against pathogen entry. The VD strain conidia were incubated in separate tubes (2.0 $\times$ 20 cm) with Czapek's agar (1×10^6 per tube; 2.0–2.5 cm columns of media) for 10 days. A warm agar solution was then added to the tubes, and a single sterilized seed was placed in this layer in each tube for each treatment. As the seeds germinated, the roots developed until they reached the layer containing fungal conidia. The experiments were conducted in three biological replicates, each independently repeated three times. Root samples were harvested from 7-day-old cotton seedlings following a 48-h exposure to VD conidia for analysis. The quantification of chitin-specific Pos was performed using the sandwich enzyme-linked immunosorbent assay (ELISA) format on 96-well plates, adapting the methodology previously detailed by Khashimova et al. [44]. Briefly, a 10-fold dilution of the plant extract from the indicator variety was added to the wells of polystyrene microplates precoated with antibodies against chitin-specific PO. A 10-fold dilution of the hybrid plant extract was added to the adjacent wells. A conjugate of rabbit immunoglobulin and alkaline phosphatase (1:1000 dilution) was applied and incubated for 1 h at 37°C. Following each incubation, the wells were washed three times with 0.01 M phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 to eliminate nonspecifically bound components. For spectrophotometric detection of the antigen–antibody complex, p-nitrophenyl phosphate in diethanolamine buffer with 0.002 M MgCl₂ was used as the substrate. After 20 min of

incubation at room temperature, the reaction was stopped by adding 50 μ L of 1 N NaOH. Absorbance was measured at 405-nm wavelength. A result was considered positive if $A_{405} > 2A_{0405}$; otherwise, it was considered negative, where A_{405} is the absorbance of the sample and A_{0405} is the absorbance of the indicator varieties (C-6524, Buxara-6).

2.7 | Microscopy Method

The penetration of the mycelium of VD fungus into cotton root cells was studied using microscopy. VD strain conidia and cotton seedlings were grown as described above, in separate tubes (2.0 $\times$ 20 cm) with Czapek's agar (1×10^6 per tube; 2.0–2.5 cm columns of media) at room temperature for 10 days. Seedling roots were harvested after exposure to VD conidia for 48 h. Fungal structures were observed following staining with Trypan Blue dye according to the Cold Spring Harbor protocol [45]. Cytological studies were performed using a CYANScope CM001 binocular optical microscope (Cypress Diagnostic, Hulshout, Belgium), and microphotography was performed using a MicroCam digital camera (San Rafael, CA, USA). All infection assays were performed at least three times.

2.8 | Determination of H₂O₂ Content

The H₂O₂ content was determined using the method described by Alexieva et al. [46], based on a spectrophotometric assay involving a reaction with potassium iodide (KI). Each sample consisted of 10 plants with three replicates. Briefly, 0.5 mL of leaf extract supernatant (prepared in 0.1% (w/v) trichloroacetic acid, TCA) was mixed with 0.5 mL of 100 mM potassium phosphate buffer (pH 7.0) and 2 mL of 1 M KI reagent (freshly prepared in double-distilled water). A blank control was prepared using 0.1% TCA instead of the leaf extract. The reaction mixtures were incubated in the dark for 1 h at room temperature, and the absorbance was measured at 390 nm using a spectrophotometer. The H₂O₂ concentration was determined using a standard curve generated using known concentrations of H₂O₂.

2.9 | Field Experiment

The best complex interlinear hybrids of F₇–F₁₁ were studied in the field by sowing after equalization in the breeding nurseries. The laboratory lines L-101, L-102, L-103, L-104, L-105, L-106, L-107, and L-108 were used as the maternal parents for hybridization, and paired interlinear hybrids of the fourth generation, such as L-101 $\times$ L-108, L-105 $\times$ L-106, L-103 $\times$ L-106, L-105 $\times$ L-108, and L-104 $\times$ L-108, were used as paternal forms.

A total of 40 direct, complex, and interlinear hybrids were generated. The C-6524 and Buxara-6 cotton varieties were used as standards for resistance to VD and FOV, six complex interlinear hybrids, one variety line, and one intervariety hybrid were studied. All the above-mentioned field experiments were conducted with a natural mixed-infection background of VD and *Fusarium oxysporum* (race 3).

For each breeding family of F₇–F₁₁, a significant trial sample comprising selected individuals and plant collections was prepared. Cotton seeds were obtained from the plants of F₃–F₁₇ families with 25 bolls. Individual selections were made from the best plants, and the best family was collected from among the families. Wilt-resistance dynamics and plant morphological

assessments were performed 15 and 20 days before cotton harvest, respectively. Disease severity was determined for individual plants, as previously described by Hillocks [3]. All variation series were prepared based on the actual data of the studied features.

2.10 | Statistical Analysis

All quantitative data were subjected to rigorous statistical evaluation. The analysis methodology followed the established procedures outlined by Maxwell and Delaney [47] as a guiding framework. For all primary breeding parameters evaluated (e.g., resistance scores and yield components), the central tendency of each feature was quantified by calculating the arithmetic (M). Precision and variability were thoroughly assessed by computing the standard error of the mean (SE), standard deviation (SD), and coefficient of variation (CV%). Crucially, these calculations were based on a robust study population of 30 replicates ($n = 30$) for each individual data point.

All biochemical experiments were performed using three independent biological replicates. Results from these assays are presented as the mean $\pm$ SE. To assess the statistical significance among the various cotton lines and control groups, the data were subjected to a one-way analysis of variance (ANOVA). Where the ANOVA indicated significant overall differences, multiple comparisons were performed using Tukey's Honestly Significant Difference (HSD) post hoc test. All differences were deemed statistically significant at a (probability level (p value) less than 0.05.

3 | Results

Cotton plants, especially those in the seedling phase, were infected with pathogens of the genera *Fusarium* and *Verticillium*, which are both part of the same pathocomplex, according to a phytopathological study conducted in some districts of the provinces of Bukhara, Tashkent, Surkhandarya, and Kashkadarya. The pathogens listed in Table 2 were obtained from the collection at the Institute of Genetics and Experimental Plant Biology (IGPEB) to study resistance to *Fusarium*.

In four districts of the Bukhara region, 42 fungal isolates from the genus *Fusarium* were isolated from soil, seeds, and various parts of cotton plants during the vegetative period, primarily during the shoot emergence phase. It was determined that the complete destruction of Buxara-6 cotton seedlings on some farms in 2011 was caused by the pathogen *F. solani*. The pathogen was identified using both mycological and molecular diagnostic methods. Laboratory studies included the isolation and identification of the pathogen based on its morphological characteristics, as well as PCR diagnostics to confirm the presence of *F. solani* [48].

In three districts in the Tashkent region, 38 isolates of fungi of the genus *Fusarium* were isolated from cotton plants, mainly those in the seedling phase, between 2012 and 2014. The species isolated included *F. sporotrichioides*, *F. fujikuroi*, *F. solani*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Fusarium oxysporum*, *F. verticillioides* (*F. moniliforme*), and *F. proliferatum*.

In the CBSPARI field plots, two isolates of the *Verticillium* genus (one and two races) were isolated from plants and soil during the vegetative period.

In the process of monitoring cotton crops in 2015–2016, 29 isolates of the genus *Fusarium* Lk. were detected in two southern areas of the Surkhandarya region. The species isolated included *Fusarium oxysporum*, *F. solani*, *F. verticillioides*, *F. proliferatum*, and *F. equiseti* [48].

3.1 | Field and Laboratory Assessments of Disease Resistance of Elite Cotton Varieties and Hybrid Combinations to Collector Strains of *Fusarium* spp.

F. solani (FS) strains No. 473 and No. 519 showed some pathogenicity to the Namangan-77 variety, while *F. solani* strains No. 319, 379, 422, and 520; *F. fujikuroi* strains No. 502 and 509, and *F. sporotrichioides* strain No. 404 showed pathogenicity ranging from 4.00 in strain 319 (FS) to 4.92 in strain 520 (FS) (Figure 1).

All the tested strains were pathogenic to Namangan-77. Strains No. 502 (*F. fujikuroi*, FF) and 519 (FS) were less virulent to C-6524 than the other strains, while *F. solani* strains No. 319, 379, 422, 473, and 520; *F. sporotrichioides* strain No. 404; and *F. fujikuroi* strain No. 509 proved to be very virulent, with mean values of pathogenicity ranging from 4.23 in strain No. 379 (FS) to 4.93 for strain No. 422 (FS) (Figure 1).

F. solani strains, *F. fujikuroi* strains 319, 422, and 519, *F. fujikuroi* strains 502 and 509, and *F. sporotrichioides* strain 404 exhibited medium virulence toward the average hybrid CBRI_3. The remaining strains were classified as weakly virulent, with mean pathogenicity values ranging from 0.73 for isolate No. from 379 to 1.33 in isolate No. 473 (Figure 1).

F. solani strains No. 319, 422, and 473. *F. fujikuroi* strain No. 509 and *F. sporotrichioides* strain No. 404 was moderately virulent to the hybrid CBRI_1. The other isolates were slightly virulent, with pathogenicity values ranging from 1.30 for strain No. 502 (FF) to 1.57 in isolates No. 379 and 519 (FS) (Figure 1).

F. solani strain No. 319, *F. sporotrichioides* strain No. 404, and *F. fujikuroi* strain No. 502 was moderately to highly virulent toward the hybrid CBRI_4. The remaining isolates were classified as slightly virulent, with average pathogenicity values ranging from 0.60 for strain No. 520 (FS) to 1.80 for strain No. 422 (FS) (Figure 1).

The variability of several economically valuable traits in elite cotton varieties and complex interlinear hybrids (F_7 – F_{11}) were assessed within a naturally infected wilt background.

All field experiments were conducted on a natural wilt background to determine the number of pathogens. Soil samples were collected from layers of sowing areas 0–10, 10–20, and 20–30 cm thick in spring, summer, and autumn. In 1 g of soil on a natural wilt background, 39,583 fungal colonies were found (0–10 cm) (Table 4).

As a result of field evaluations and statistical analysis in 2019, it was possible to reveal some genetic peculiarities and identify significant hybrids F_7 – F_{11} through selection (Table 5). Simultaneously, the best families, that is, the best hybrids, from previously conducted research were used for breeding in 2019 under field conditions. Special attention was paid to determining the resistance to VD and FOV.

When the SD (δ) values were examined, hybrids CBRI_5, CBRI_6, and CBRI_8 had the lowest values, ranging between

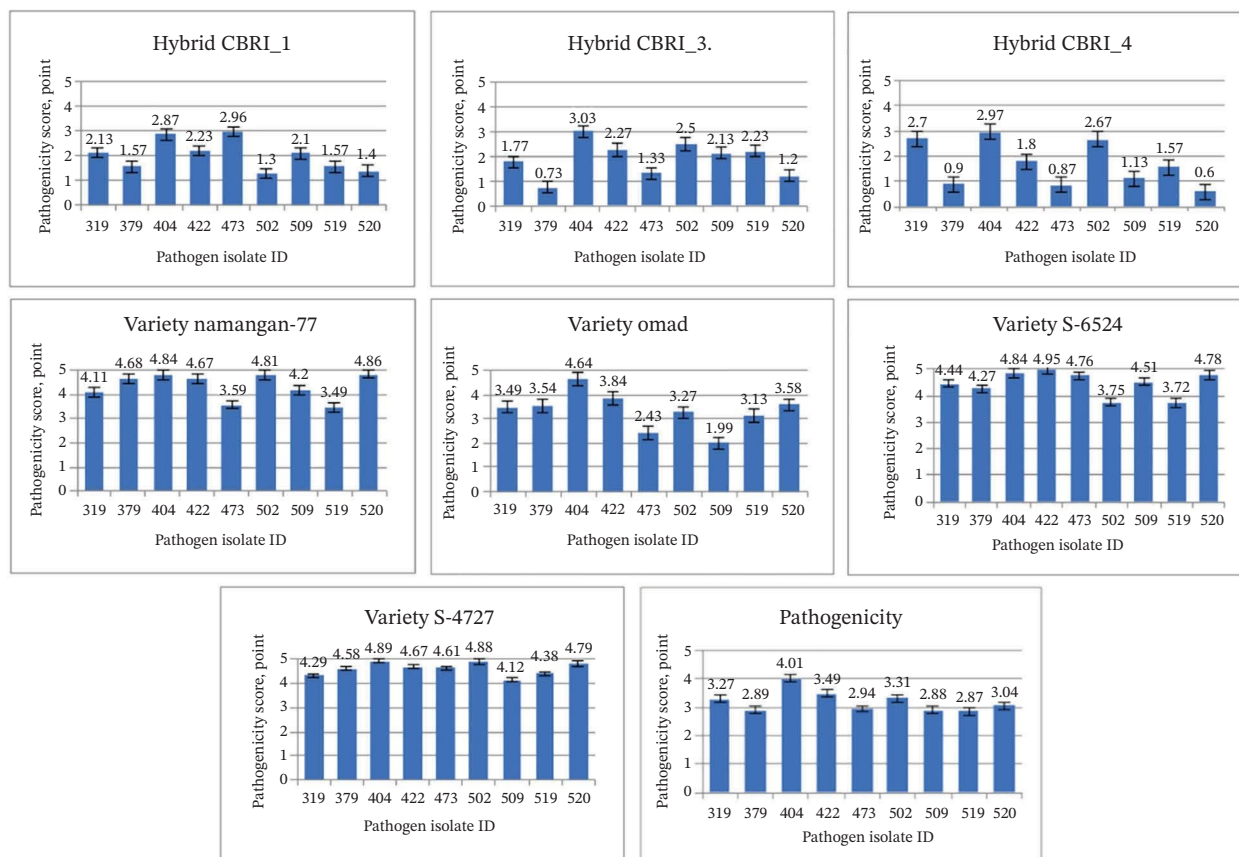


FIGURE 1 | Resistance to isolates of pathogens of the genus *Fusarium*.

TABLE 4 | Distribution of fungi by soil depth under field conditions.

No.	Name of the place	Soil depth (cm)		
		0–10	10–20	20–30
1	Naturally infected wilt background	39.583	36.416	28.125

TABLE 5 | Variability of the feature resistance to *Verticillium dahliae* and *Fusarium oxysporum* within a naturally infected wilt background in complex interlinear, varietal, and intervarietal hybrid combinations of F₇–F₁₁.

Variety indicator, hybrid				
No.	combination	<i>M</i> ± <i>SE</i>	<i>SD</i>	<i>CV</i> %
1	C-6524 (variety-indicator)	2.93 ± 0.2	1.19	40.68
2	Buxara-6 (variety-indicator)	2.85 ± 0.27	1.59	55.71
3	CBRI_1	2.93 ± 0.2	1.19	40.68
4	CBRI_2	2.85 ± 0.27	1.59	55.71
5	CBRI_3	0.76 ± 0.06	0.75	98.58
6	CBRI_4	0.80 ± 0.06	0.78	98.69
7	CBRI_5	0.60 ± 0.02	0.40	67.30
8	CBRI_6	0.61 ± 0.02	0.35	57.57
9	CBRI_7	0.67 ± 0.02	0.58	86.58
10	CBRI_8	0.52 ± 0.01	0.17	32.27

Note: According to the results presented in Table 5, the families of F₁₁ hybrids, such as CBRI_3, CBRI_6, CBRI_7, and CBRI_8, were of particular interest, as their resistance values ranged from 0.52 to 0.67. However, the indicator varieties C-6524 and Buxara-6 were equal at 2.93 and 2.85 points, respectively.

0.17 and 0.40, demonstrating that this trait had low variability in the selected family.

3.2 | Identification of Hybrids Resistant to *Fusarium* and VD Using DNA Marker Selection Technology (MAS)

DNA was extracted from 96 harvested cotton samples derived from the young leaves of naturally infected plants, which were created through complex interlinear, line-grade, and intergrade hybridization of cotton lines. Genetic analysis was performed to identify prospective plant samples potentially resistant to *Fusarium* and VD.

To identify meaningful DNA polymorphisms in the genotypes investigated, PCR settings were optimized, followed by primer pair screening with randomly selected samples. PCR conditions that generated the maximum quantity of polymorphic DNA products were selected. Six pairs of primers were selected as the most promising DNA markers for subsequent studies (Table 6).

The primary analysis established that the hybrid CBRI_3 was the most promising hybrid. PCR using three primer pairs (Figure 2) identified candidate loci associated with wilt resistance in the largest number of hybrid plants. Families 5 and 7 were the most promising genotypes for this hybrid, as they had selective loci for all three markers. Family number 2, which has two out of three selective loci, can also be considered a promising genotype (Figure 2). According to the PCR analysis, the CBRI_3 hybrid genotypes possessed BNL4003 (145 bp), CM209 (140 bp), and

TABLE 6 | The most promising DNA markers used in the study.

Marker name	Primer 1	Primer 2
BNL4003	CCTTAAAGAGAGGATATACACAAACG	AAGGGCGGAGAAAGTAGGAG
TMB0161	GATCACCCAAGAGCTCACAC	CCATTTAGGGTCCAAAATCATC
BNL3090	GAAATCATTGGAAGAACATATACTACA	TTGCTCCGTATTTTCCAGCT
TMB0307	GGAGCCTGGGTCTCTAGCTT	ACGGTGGTCTGGTGACTGA
TMB1660	GCATTGAATAATACTGGCTAAGAGC	CAATAACAAATTTAGCCCATCG
CM209	GAGAGCCGAGGACCCAGAAG	GTCAGGTTGGTTGTTGG

Note: Genotyping of 96 DNA samples from cotton plants bred through interlinear, linear, and intergrade hybridization was then carried out to identify genotypes potentially resistant to *Fusarium* and *V. dahliae*.

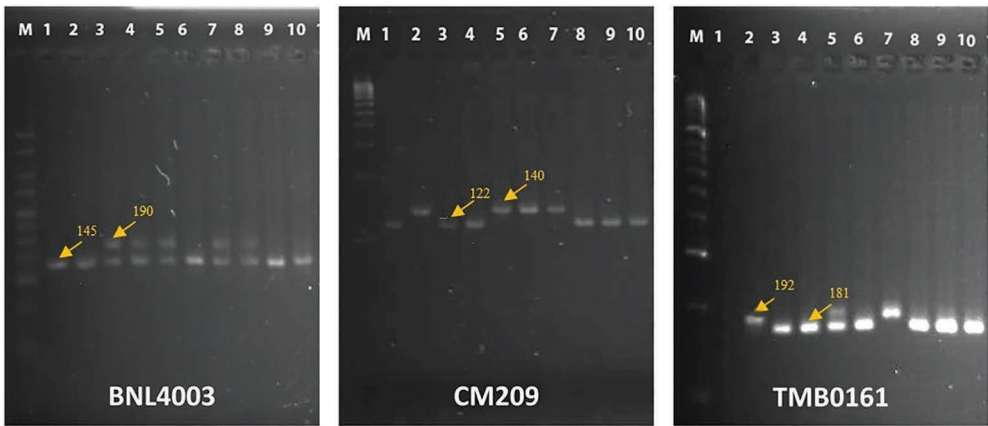


FIGURE 2 | Agarose gel electropherogram showing SSR marker analysis for *Fusarium* wilt resistance in 10 individual genotypes (lanes 1–10) of the hybrid line CBRI_3. Lane M 50 bp and M 100 bp molecular weight markers. The analysis utilized three SSR primers previously identified as being linked to resistance: BNL4003, CM209, and TMB0161. The arrows indicate the presence of resistance-associated alleles at their expected sizes (145, 140, and 192 bp). Although susceptible and resistant checks were used in the overall study, this gel is a representative image showing polymorphism within the CBRI_3 hybrid line.

TMB0161 (192 bp) marker alleles associated with FOV resistance [43] (Figure 2).

3.3 | Biochemical Analysis of Prospective Genotypes

The stability and susceptibility of plants are inheritable features that can be displayed under extreme circumstances. In response to disease infection, cotton plants enhance certain biochemical pathways to resist pathogen invasions.

It is important to determine whether the impact of the pathogen depends on changes in PO enzyme activity and the accumulation of H_2O_2 to understand the processes that occur during the early stage of VD interaction with cotton and, later, in the establishment of protective responses against infection in various cotton genotypes.

VD isolated from the soil was used as a stress factor in this study. Cotton infection with VD occurs mainly through the root system, which is the primary barrier against pathogen penetration.

The study of PO activity in the seedling roots of various cotton hybrids showed that when interacting with phytopathogens in all experimental variants, except for Buxara-6, C-6524, and the hybrids CBRI_2, CBRI_4, CBR_5, and CBRI_7, PO activity decreased by 1.07–1.73 times from the control level (Figure 3). As

shown in Figure 3, the PO activity of the cotton hybrid CBRI_1 was 70.4 units/mg, which was considerably close to the control level of 69.2 units/mg. The hybrid CBRI_6 also had similar experimental and control enzyme activity values, of 96.6 units/mg and 97.2 units/mg, respectively.

Analysis showed that in the two most resistant hybrids, CBRI_3 and CBRI_8, the PO enzyme activity increased significantly, with levels 1.6 and 1.3 times greater than those in the control group, respectively.

Early plant defense responses against pathogens involve the generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) [49]. H_2O_2 serves a dual function: it acts as a signaling molecule at low concentrations and provides a direct biocidal effect at higher, toxic concentrations [50, 51]. Furthermore, the interaction of H_2O_2 with PO is critical for reinforcing the cell wall through lignification, a known defense mechanism [52]. Consistent with this role, the H_2O_2 content in cotton roots showed a sharp increase 48 h after inoculation (Figure 4).

As shown in Figure 5, the highest H_2O_2 content was observed in the hybrids $CBRI_3 > CBRI_8 > CBRI_7 > CBRI_6 > CBRI_5 > CBRI_4 > Buxara-6$, where their values exceeded those in the control by $9.3 > 5.6 > 4.2 > 3.8 > 2.5 > 2.4 > 1.8$ times, respectively. The accumulation of H_2O_2 may lead to oxidative stress in the cotton seedlings. The ability of the

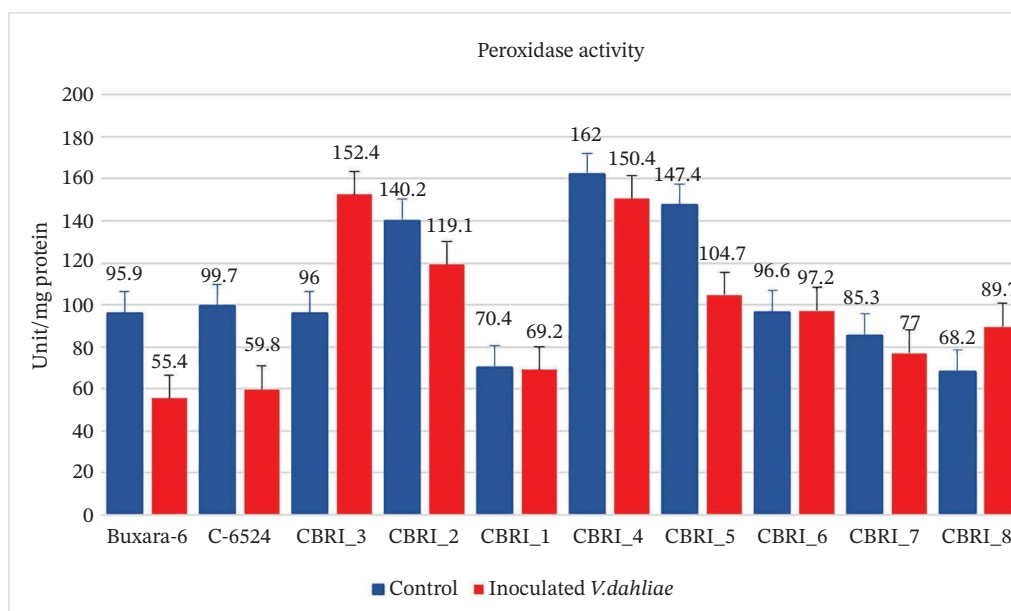


FIGURE 3 | Peroxidase activity in the roots of 7-day-old seedlings of various cotton hybrids after 48 h of exposure to *V. dahliae* conidia.

Verticillium fungal pathogen to systematically suppress the activity of enzymes involved in the formation of APA at the early stages of pathogenesis in cotton hybrids should be assumed to be one of the factors in the overall strategy aimed at overcoming the plant host's protective systems. The aforementioned results were confirmed by cytochemical studies in the roots of various cotton hybrids inoculated with VD (Figure 5).

Root tissues from C-6524 and the hybrids CBRI_2 and CBRI_4 seedlings were fixed and stained in lactophenol with 0.5% trypan blue, and as shown in Figure 5, the necrosis zone was dyed an intensive blue color. Both plant and intruding pathogen cells die, causing necrotic damage in the hypersensitive reaction. Such plant cell death in the zone of microorganism localization is essential, as it causes the death of the pathogens and prevents them from spreading further into the plant. Mycelial infiltration was not observed in CBRI_3 seedlings. Both control and inoculated seedlings exhibited significant levels of PO enzyme activity, but the H_2O_2 content in inoculated seedlings exceeded

that of the control by 9.3 times. In the seedlings of the hybrids CBRI_1, CBRI_5, CBRI_6, and CBRI_8, the mycelium of the fungus did not penetrate the deep layers of the root parenchyma but instead remained on the surface layers of the root tissue. Most likely, the high H_2O_2 level accumulated in these hybrid lines prevented the fungus from penetrating the deep cell layers. After inoculation, the mycelium of the fungus developed minimally in the hybrid CBRI_8 seedlings. This observation was corroborated by the absence of mycelial branching, a 1.3-fold increase in mycelial activity, and a 5.6-fold increase in H_2O_2 concentration compared to the control.

Therefore, it is highly probable that H_2O_2 contributed to the development of the observed hypersensitivity reactions, which likely activated the expression of protective protein genes to suppress further growth of the fungal mycelia, warranting further investigation. These findings demonstrate the direct participation of PO in necrosis formation and the increase in H_2O_2 concentration as protective responses of cotton to pathogen

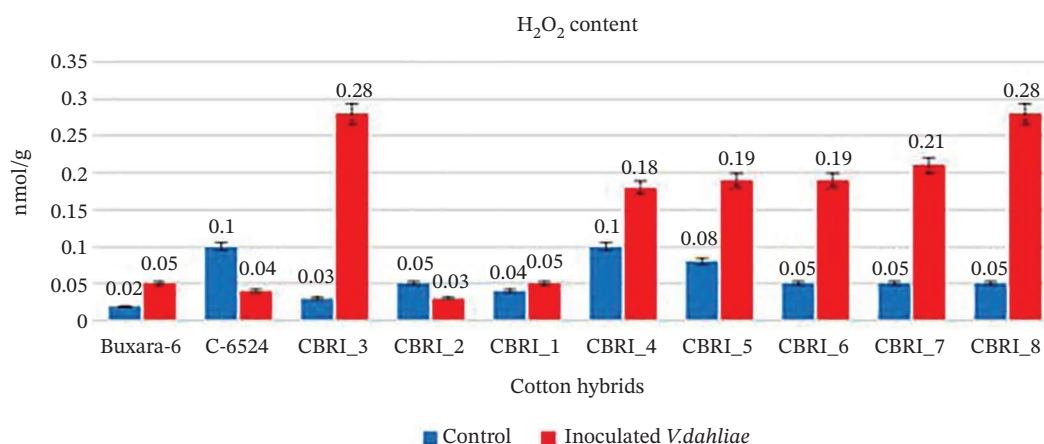


FIGURE 4 | Hydrogen peroxide content in the roots of 7-day-old sprouts of various cotton hybrids after 48 h of exposure to *V. dahliae* conidia.

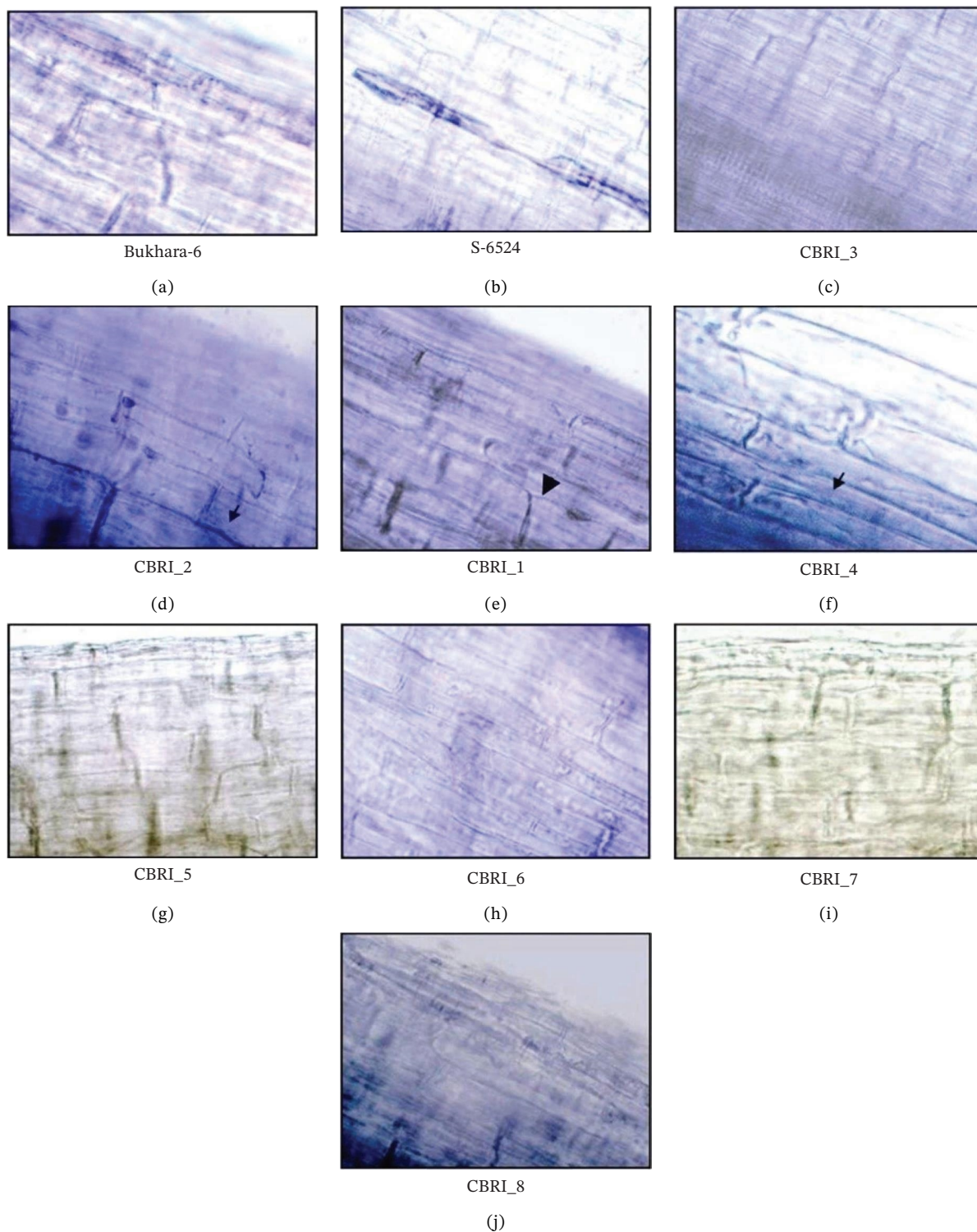


FIGURE 5 | Cytochemical study of seedling roots of various cotton hybrids (fixation in lactophenol with 0.05% trypan blue). Arrows indicate the location of *V. dahliae* mycelia.

invasion. The higher the H_2O_2 concentration and PO enzyme activity, the greater the resistance of the cotton lines.

4 | Discussion

To date, the most effective and feasible way to combat VW and FW disease is the selection of cotton varieties resistant to the

pathogen using traditional breeding and transgenic technologies [53–55]. In this study, 8 complex cotton hybrids and 10 fungal isolates were used to identify promising hybrids resistant to VW and FW. Two previously screened cultivars were used as resistance benchmarks in the field experiments. Biochemical, molecular, and microscopic analyses, as well as repeated field experiments, were conducted to obtain meaningful results. This

study involved the development of cotton hybrids resistant to VW and FW through seedling-stage screenings. Rapid and reliable methods are required to screen for fungal pathogen-resistant germplasm that cotton breeders can employ in their breeding programs.

As a result of field and laboratory analyses, resistance patterns among the hybrids were identified. This approach made it possible to recommend the best hybrids for further breeding studies in the future. The hybrids with the most significant potential in terms of resistance to VD and FOV from a breeding perspective were CBRI_3 ($M = 0.6$), CBRI_6 ($M = 0.52$), CBRI_7 ($M = 0.57$), and CBRI_8 ($M = 0.54$).

Molecular analysis revealed that six primer pairs were the most promising DNA markers for identifying cotton lines resistant to FOV and VD. Based on the highest number of plants with identified candidate loci associated with indicators of wilt resistance, PCR with three primer pairs (BNL4003, CM209, and TMB0161) indicated that the hybrid CBRI_3 was the most potentially resistant germplasm. The markers associated with resistance QTL may facilitate the use of VW resistance genes to improve breeding programs for cotton.

PO participates in the polymerization of monolignols into lignin and strengthens the cell wall following pathogen attack and wounding [56]. VD infection caused considerable changes in the production of PO enzyme. Biochemical analysis using phytoimmunity-indicating enzymes, particularly PO, and cytochemical analyses of the roots of several cotton hybrids indicated that CBRI_3 and CBRI_8 were the most resistant to VD and thus the most interesting for breeding purposes. The increased enzyme activity allowed the plant to reinforce its cell walls in response to infection and engage in processes that prevented the multiplication of the fungus' infectious structures.

The decrease in PO activity observed in other hybrid lines may stem from enzyme inhibition caused by the excessive buildup of H_2O_2 (hyperproduction) as fungal structures spread through cotton tissues. Resistance to vascular pathogens, such as *Verticillium*, is generally believed to depend mainly on the mechanical isolation of the pathogen within the host's vascular system during crucial phases of colonization [57]. This mechanism relies on lignin, which forms a structural barrier to prevent the outward diffusion of fungal toxins and extracellular enzymes, thereby restricting pathogen spread. For this cell wall defense mechanism to be effective, it is essential that lignification occurs rapidly and promptly, preceding the penetration of the cell wall by pathogen hyphae, thus ensuring the stability of the protective barrier against pathogen invasion. Consequently, the defensive response of cotton seedlings to VD relies heavily on cell wall fortification via the deposition of lignin and lignin-like polymers, which is preceded by the activation of the transcription of enzymes responsible for their production.

The introduction of new varieties will be accompanied by the evolution of new races of the fungus. According to Ranga et al. [58], scientists are currently aiming not only to find donors of resistance to individual races but also to create donors of complex resistance to different races of the fungus through stepwise hybridization and accumulation of genes of resistance to different races of the fungus in the genotype of new varieties.

Through our comprehensive molecular and biochemical analyses, the L-1077 line was conclusively identified as exhibiting strong resistance to both VD and FOV among all tested breeding materials. This successful line was rigorously isolated from the complex interlinear hybrid combination CBRI_3 ($F11[F6(L-101 \times L-105) \times L-106]$). The L-1077 selection is notable for producing fiber that meets the demanding type IV fiber quality standards, delivering high fiber yields, and maintaining robust resistance to both VD and FW. Consequently, the Intellectual Property Agency of the Republic of Uzbekistan has issued an application for patent for this line, designating it under the cotton variety name C-6602 (NAP 2020 0007 from 31.01.2020).

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Disclosure

A preliminary version of this study was presented as an abstract at the Plant Cell, Tissue Culture *In Vitro* IV conference (<https://viscea.org/conference/plant-cell-tissue-culture-in-vitro-iv/>). The present manuscript is a substantially expanded and revised version and includes original data and analyses that have not been published previously.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The original experimental dataset used in this study is available from the corresponding author and the first author upon request.

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