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Attenuated Isolate *Gibellulopsis nigrescens* Vn-1 Enhances Resistance against *Verticillium dahliae* in Potato

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Abstract: Potatoes are among the four most important staple crops worldwide. *Verticillium* wilt in potatoes caused by *Verticillium dahliae* is a devastating disease that is difficult to control. To identify potential avenues for disease control, the pathogenicity of 72 *V. dahliae* isolates was tested here. We also tested the resistance to the most virulent isolate (Vd-36) induced by the attenuated isolate *Gibellulopsis nigrescens* Vn-1 in potatoes. Induction of *Verticillium* wilt resistance in potatoes was strongest when using attenuated isolate Vn-1 to inoculate potatoes with a spore suspension concentration of 1×10^6 conidia mL^{-1}, followed by infection with isolate Vd-36 at 5 d intervals. After incubation of potatoes with the attenuated isolate Vn-1 followed by isolate Vd-36, reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) were produced and accumulated in potato leaves 12 h post-inoculation. The changes in respective defense enzymes, except phenylalanine ammonia-lyase, were consistent with the changes in ROS and H_2O_2 levels. Furthermore, the content of salicylic acid (SA) in inoculated plants was higher than that in the control, and biosynthesis-related genes *StNPR1*, *StPR1b*, *StPR2*, *StPR5* were activated. However, there was no significant difference in the jasmonic acid and ethylene (JA/ET) content between the treatment and control groups. These results demonstrated that the attenuated isolate Vn-1 enhanced resistance to *Verticillium* wilt by inducing the SA signalling pathway and weakly activating the JA/ET signalling pathways in potatoes.

Keywords: *Gibellulopsis nigrescens* Vn-1; *Verticillium dahliae* Vd-36; *Verticillium* wilt; induced resistance; salicylic acid; jasmonic acid and ethylene; signalling pathway

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1. Introduction

Potato (*Solanum tuberosum* L.) is a dicotyledonous plant belonging to the Solanaceae family and is one of the four major food crops cultivated worldwide [1]. *Verticillium* wilt is a major economic disease that occurs in commercial agriculture [2]. Yield losses of potato crops due to *Verticillium* wilt are generally in the range of 10–15% but may reach 50% [3–5]. The main causal agents of *Verticillium* wilt in potatoes include *Verticillium dahliae* and *Verticillium albo-atrum*, which predominantly invade the xylem of their host plants and disrupt water transport, eventually leading to the wilting of host vascular bundles [2]. Additionally, *Verticillium nigrescens* and *Verticillium tricorpus* may infect potatoes and other plants; however, they may be pathogenic or non-pathogenic [6–8]. According to Zare [9], *V. nigrescens* belongs to the genus *Gibellulopsis* and should thus be named *Gibellulopsis nigrescens*. Lower leaves of potato plants infected with *V. dahliae* turn yellow and necrotic showing vascular bundle discoloration, stunting, and wilting. In contrast, *G. nigrescens* is saprophytic and weakly invasive, and leaves do not show discoloration or wilting following infection [10,11].

Two-branch systems are typically triggered when plants are attacked by pathogens which produce various commonly conserved molecules collectively known as pathogen-related molecular patterns (PAMPs), including flagellins, lipopolysaccharides, and peptides. PAMPs are detected by host-cell PAMP recognition receptors, which induce a

series of cellular physiological and biochemical changes to counteract the pathogens. Such resistance is termed pattern-triggered immunity (PTI). Effector-triggered immunity (ETI) is a further resistance system, which is activated by the recognition of secreted microbial effectors by intracellular receptors and induces strong, specific, and localized immune reactions [12,13]. Plant systemic acquired resistance (SAR) is induced by pathogenic bacteria, and induced plant systemic resistance (ISR), triggered by non-pathogenic microorganisms, is an important defense mechanism of microbial-mediated plant resistance [14]. Previous studies suggested that SAR depends mainly on the salicylic acid (SA) signalling pathway. After pretreatment of plant tissues with virulent, attenuated, non-pathogenic microorganisms, and synthetic chemicals, unpretreated parts of the plant and the entire plant can acquire resistance [15–17]. However, ISR typically relies on the jasmonic acid (JA) and ethylene (ET) signalling pathways, which are activated by non-pathogenic soil microorganisms to protect their host from necrotising pathogens and insects [18–20]. β-1,3-Glucan, Flg22, and Nep1 in PAMPs are also involved in the JA/ET and SA signalling pathways [21–23]. In tomato plants, the endophytic bacterial strain EBS05 induces resistance to tomato yellow leaf curl virus by eliciting a synergism between the SA and JA signal transduction pathways [24].

China is the largest potato producer in the world, and Inner Mongolia has become an important production base for seed, commercial, and special potatoes. However, the occurrence of potato *Verticillium* wilt has become the main factor that affects potato yield and limits the development of the potato industry owing to continuous potato cropping [25]. Extensive persistence of *V. dahliae* in soil also makes it difficult to avoid recurring plant infection and reducing *Verticillium* propagules in soil through soil fumigation with various chemicals is expensive and adversely affects beneficial soil flora, human health, and the environment [26,27]. Although crop rotation can reduce the effect of soil-borne diseases on crop plants, it is not sufficiently effective for *V. dahliae*, which exhibits long latency periods in soil [2]. Thus, new methods for controlling *Verticillium* wilt are required. In our previous study, sunflowers developed resistance to *Verticillium* wilt after inoculation with the attenuated isolate *G. nigrescens* Vn-1 and subsequent challenge inoculation with the highly virulent isolate *V. dahliae* V33 [28]. Thus, we here explored potential resistance to *Verticillium* wilt induced by the attenuated isolate *G. nigrescens* Vn-1. The objectives of this study were as follows: (i) 72 *V. dahliae* isolates were selected from across China, and the most virulent isolate was selected by determining pathogenicity. (ii) Resistance induced by attenuated isolate Vn-1 to the virulent isolate *V. dahliae* in potatoes was tested. (iii) The mechanisms through which isolate Vn-1 induced host resistance to potato *Verticillium* wilt was examined to identify potential interactions between pathogen and hosts and to help identify novel biological control methods for potato *Verticillium* wilt.

2. Materials and Methods

2.1. Plants and Pathogens

The potato (*Solanum tuberosum* L.) cultivar Favourite (susceptible) [29] seeds and plantlets were purchased from Wuchuan SaiFeng Potato Seed Industry Co., Ltd., Hohhot, Inner Mongolia, China.

G. nigrescens Vn-1(GenBank accession: OP913393) and 72 *V. dahliae* isolates (Table S1) were procured from the Fungus Preservation Collection of the Inner Mongolia Agricultural University (IMAU), China. Vn-1 was isolated from infected sunflower plants and was used to inoculate potato plants and test the induction of resistance against *Verticillium* wilt in potatoes. Pathogens were isolated and identified as follows:

Stems of sunflowers and potatoes showing symptoms of *Verticillium* disease were collected. The stems were peeled and cut into small pieces (0.3–0.5 cm long) which were surface-sterilized by dipping in a bleach solution of 5% NaClO for 3 min. The sterilized sections were then rinsed in sterile distilled water for 2 min, soaked in 70% ethanol for 2 min, and were subsequently washed again with sterile distilled water for 60 s. The stem sections were placed in Petri dishes containing potato dextrose agar (PDA) and

were incubated at 25 °C in an incubator under dark conditions. The single-spore method was used to purify fungal colonies growing out of diseased tissues. Genomic DNA from the cultures was extracted by Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech Shanghai Co., Ltd., Shanghai, China). In order to characterize the species of pathogens, the universal primer sets ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [11] were used for amplifying the 18S rDNA intron. Then, sequencing was carried out by Beijing Genomics Institution (BGI). Finally, the sequencing results were uploaded to GenBank for accession numbers and analyzed by BLASTN in NCBI database. These cultures were used for morphological and molecular analyses. The identified pathogens were stored at −80 °C.

Fungi preserved at −80 °C were cultured on PDA medium for 15 d and were then cultured in potato dextrose broth on a rotator at 180 rpm and at 25 °C for 7 d in the dark for conidia production. The concentrations of the conidial suspensions were adjusted using a hemocytometer with sterile distilled water. We prepared different concentrations (1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 conidia mL^{-1}) of conidial suspension of Vn-1 to identify the optimum concentration to induce resistance against *Verticillium* wilt in potatoes.

2.2. Isolate Virulence Screening

Concentrations of conidial suspensions of 72 *V. dahliae* isolates were adjusted to 1×10^7 conidia mL^{-1}. Potato plantlets in vitro were grown hydroponically for 7 d, followed by transplanting to small transparent tubes containing ceramsite (Shanghai Hongjun Gardening Supplies Co., Ltd., Shanghai, China), after which they were inoculated with *V. dahliae* isolates using spore suspensions to assess pathogenicity based on disease symptoms [30]. We then calculated the disease indices (DIs) of potatoes at 15 days post-inoculation (dpi) as follows, using four rating scales, as described previously [16]:

0 = no disease symptoms;
1 = several chlorotic leaves in less than 25%;
2 = partial necrosis and wilting, 25–50% of the diseased leaves showed symptoms;
3 = more than 50% of the diseased leaves showed symptoms, with a few leaves shed;
4 = severe wilting or death.

The DIS was then calculated as follows [16]:

$$DI = (\sum Xi \times Yi / Xmax \times \sum Yi) \times 100\%$$

Xi: rating scale; Xmax: maximum rating scale; Yi: number of diseased plants of each rating scale.

Three replicates were used, with 30 potato plants, each.

2.3. Vn-1-Induced Immune Response to Verticillium Wilt in Potato

2.3.1. Optimal Interval

A total of 10 groups were established. The selected *V. dahliae* isolate (SVD) was used to inoculate potatoes that were planted after 25 d in pots with nutrient soil at intervals of 1, 2, 3, 4, 5, 6, and 7 d after inoculation with Vn-1. Potatoes inoculated only with sterile water, Vn-1, or SVD were used as controls. All inoculations were performed using a concentration of 1×10^7 conidia mL^{-1}. The DI of the potatoes in each treatment and control group was calculated at 20 dpi. The experiments were conducted thrice, and 30 potatoes were collected each time.

2.3.2. Optimum Concentration

Ten groups were used. SVD was used to inoculate the potatoes at the optimal induction interval after inoculation with Vn-1. The inoculation conidia concentrations of Vn-1 were 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 conidia mL^{-1}. Potatoes inoculated only with sterile water, Vn-1, and SVD with a conidial suspension concentration

of 1×10^7 conidia mL^{-1} were used as controls. The DI was calculated at 20 dpi. The above experiments were repeated three times with 30 potato plants per repetition.

2.3.3. Reactive Oxygen Species (ROS), Hydrogen Peroxide (H_2O_2) Accumulation, and Respective Enzyme Activities

We performed 3,3′-diaminobenzidine (DAB) staining to measure ROS levels, as described previously [31] with some modifications. The selected potato leaves collected 0, 12, 24, 48, 72, and 96 h post-inoculation (hpi) were immersed in DAB solution (1 mg mL^{-1} in 10 mM Tris-HCl at PH 6.5) overnight at 25 °C under dark conditions after filtration under complete vacuum. The leaf samples were bleached in 95% alcohol in a boiling water bath for 20 min. Subsequently, leaf samples were electronically scanned (CanoScan LiDE 400, Canon China Co., Ltd., Beijing, China) in a pure white setting.

H_2O_2 accumulation and activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and phenylalanine ammonia-lyase (PAL) were measured using respective test kits (Sino Best Biological Technology Co., Ltd., Shanghai, China). The leaf sampling time was the same as that for DAB staining.

The experiments were conducted using three replicates, with nine leaves each.

2.4. Analysis of SA, JA, and ET Signalling Pathways

Potato leaves (0.1 g) were collected and were then centrifuged at 12,000 rpm and 4 °C to obtain the supernatant. SA, JA, and ET were quantified using enzyme-linked immunosorbent assays (ELISAs) using plant an SA, JA, and ET ELISA Kit (Sinobestbio, Shanghai, China).

2.5. Relative Expression Levels of Related Defense Genes in Signalling Pathways

Total RNA was extracted from leaves using a plant RNA extraction kit (TaKaRa, China). First-strand cDNA was synthesized using the PrimerScript RT Master Mix (TaKaRa). Quantitative real-time polymerase chain reaction (qtr.-PCR) was performed using an ABI7700 Real-Time PCR system with TB Green® Premix Ex Taq™ (Tli RNaseH Plus; TaKaRa) according to the manufacturer's instructions. The PCR program was conducted as follows: 95 °C denaturation for 1 min, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 15 s. StEF1 (LOC102600107) was used as an internal control to normalize the results; qRT-PCR data were analyzed using $2^{-\Delta\Delta Ct}$ data and a one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$). All experiments were repeated at least twice in triplicates. All gene and primer details are listed in Supplementary (Table S2).

2.6. Statistical Analyses

All experiments were performed at least three times per treatment. The data were processed using SPSS Statistics software (version 25.0; IBM China Company Ltd., Beijing, China) or one-way ANOVA, followed by Tukey's test ($p < 0.05$). Values were analyzed and mapped using GraphPad Prism software (version 8.0; GraphPad Software, San Diego, CA, USA))

3. Results

3.1. Pathogenicity of V. dahliae

Pathogenicity of 72 *V. dahliae* isolates was assessed. Here, a DI< 30 indicated a weakly virulent isolate, a DI from 30 to 50 indicated a moderately virulent isolate, and a DI > 50 indicated a highly virulent isolate. Ten weakly virulent isolates were identified, among which Vd-66 was the least virulent pathogen with a DI of 17.18. There were 55 moderately virulent isolates, including Vd-16, Vd-33, and Vd-55, and 7 highly virulent isolates, of which Vd-36 was the most virulent isolate, with a DI of 65.62 (Table 1).

Table 1. Pathogenicity of *V. dahliae* isolates.

Isolate	Disease Index	Isolate	Disease Index	Isolate	Disease Index
Vd-1	40.36	Vd-25	37.31	Vd-49	43.28
Vd-2	44.83	Vd-26	40.22	Vd-50	62.26
Vd-3	38.4	Vd-27	31.93	Vd-51	33.02
Vd-4	39.46	Vd-28	32.22	Vd-52	38.34
Vd-5	43.99	Vd-29	25.07	Vd-53	34.95
Vd-6	30.32	Vd-30	28.77	Vd-54	34.76
Vd-7	29.11	Vd-31	33.33	Vd-55	40.7
Vd-8	31.27	Vd-32	36.16	Vd-56	39.04
Vd-9	18.21	Vd-33	36.57	Vd-57	41.1
Vd-10	17.28	Vd-34	34	Vd-58	39.93
Vd-11	31.08	Vd-35	44.09	Vd-59	39.82
Vd-12	30.95	Vd-36 *	65.62 *	Vd-60	46.77
Vd-13	28.87	Vd-37	50.62	Vd-61	41.76
Vd-14	31.45	Vd-38	38.18	Vd-62	40.73
Vd-15	54.24	Vd-39	42.02	Vd-63	48.13
Vd-16	40.37	Vd-40	44.57	Vd-64	43.34
Vd-17	32.82	Vd-41	38.04	Vd-65	22.06
Vd-18	40.2	Vd-42	31.09	Vd-66	17.18
Vd-19	45.23	Vd-43	36.13	Vd-67	53
Vd-20	31.18	Vd-44	31.8	Vd-68	45.93
Vd-21	28.89	Vd-45	36.31	Vd-69	52.5
Vd-22	40.9	Vd-46	40.1	Vd-70	37.02
Vd-23	28.03	Vd-47	46.98	Vd-71	51.43
Vd-24	45.35	Vd-48	35	Vd-72	30.42

Note: The asterisk indicates the most virulent isolate.

3.2. Inoculation Treatment of Attenuated Pathogen G. nigrescens Vn-1 Enhanced Potato Resistance against Verticillium Wilt

We previously demonstrated that *G. nigrescens* Vn-1 can induce resistance to *Verticillium* wilt in sunflowers [28]. To determine the resistance induced by *G. nigrescens* Vn-1 in potatoes, the occurrence of potato *Verticillium* wilt was monitored, and the DI was calculated 25 d after inoculation with the less virulent isolate *G. nigrescens* Vn-1, followed by the highly virulent isolate *V. dahliae* Vd-36 which was applied at a certain interval (1, 3, 5, and 7 d) in a pot experiment. Each pot was sprayed with 30 mL of conidial suspension at a concentration of 1×10^7 conidia mL^{-1}. As shown in Figure 1A,B, the symptoms and DI of *Verticillium* wilt on potatoes inoculated first with *G. nigrescens* Vn-1 and then with *V. dahliae* Vd-36 at intervals of 1, 3, 5, and 7 d were less severe and lower, respectively, than those of the CK3 group (only inoculating *V. dahliae* Vd-36) but more severe and higher, respectively, than those of the CK2 group (only inoculating *G. nigrescens* Vn-1). *G. nigrescens* Vn-1 can thus enhance the resistance of potatoes to Vd-36. The DI of *Verticillium* wilt was lowest at an interval of 5 d at 19.15, which indicated that the optimal induction interval was 5 d. Subsequently, a series of conidial suspension concentrations (from 1×10^1 to 1×10^8 conidia mL^{-1}) of *G. nigrescens* Vn-1 was sprayed on living potato plants 5 d before inoculation with *V. dahliae* Vd-36 using a pot experiment assay. As shown in Figure 1C, the DI of *Verticillium* wilt on potatoes inoculated with a conidial suspension concentration of 1×10^6 conidia mL^{-1} of *G. nigrescens* Vn-1 was the lowest (only 18.61), compared with that of other plants inoculated with different conidial suspension concentrations, in contrast with CK3. Therefore, the optimal inoculation concentration was 1×10^6 conidia mL^{-1}.

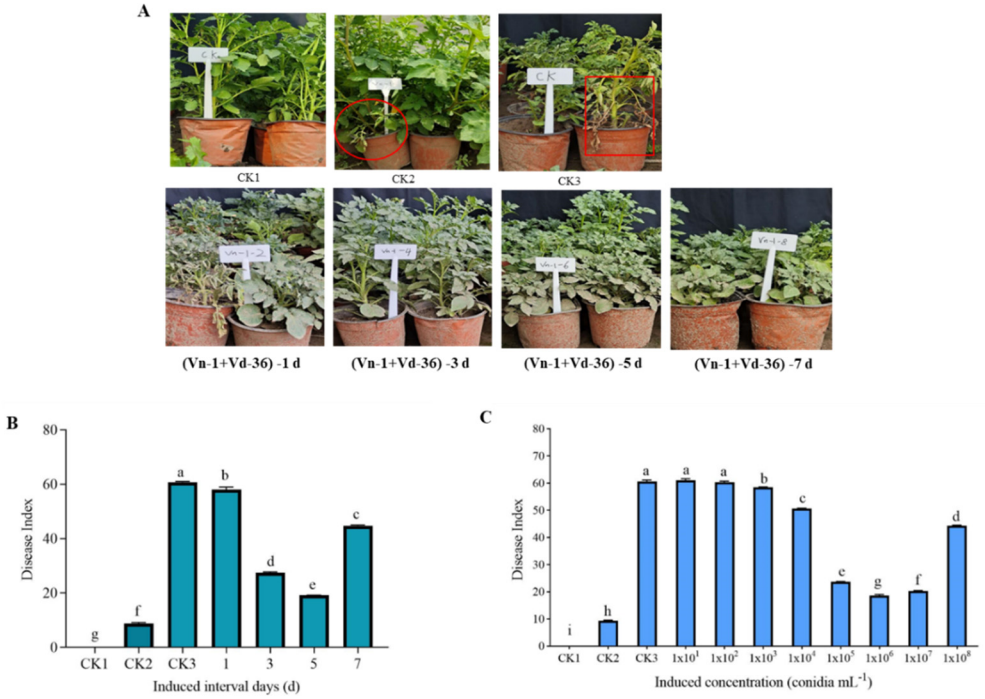

Figure 1. Less-virulent isolate *Gibellulopsis nigrescens* Vn-1 enhances potato resistance to *Verticillium* wilt caused by *Verticillium dahliae*. Disease symptoms and disease indexes of living plants assayed after 25 d in the pot experiment treated with *G. nigrescens* Vn-1 at different interval days and different conidial suspension concentrations of *G. nigrescens* Vn-1, first at 5 d post-inoculation before inoculation with *V. dahliae* Vd-36: (**A**) disease symptoms; (**B**) disease indexes of potato *Verticillium* wilt at different induced interval days; (**C**) disease indexes of potato *Verticillium* wilt at different induced conidial suspension concentrations (from 1×10^1 to 1×10^8 conidia mL^{-1}) of *G. nigrescens* Vn-1. CK1 was inoculated with sterile water, and only less-virulent isolate *G. nigrescens* and strong-virulent isolate *V. dahliae* Vd-36 were used in CK2 and CK3, respectively, as controls. Significant differences are indicated by letters above bars (Tukey's multiple range test, $p < 0.05$).

3.3. ROS Burst, H_2O_2 Accumulation, and Respective Enzyme Activities

As signalling molecules in biological processes, ROS are common secondary messengers in various cellular physiological and biochemical processes. During plant–pathogen interactions, the release of ROS in plants is assumed to be an initial response due to special oxidase activity induced by the elicitor pathogen in the host plant which produces O^{2-} and H_2O_2. H_2O_2 is the most stable and common molecular form of ROS [32,33]. To measure the accumulation of ROS and H_2O_2, we performed DAB staining and H_2O_2 content determination at 0, 12, 24, 48, 72, and 96 hpi on the leaves of four different treatments (Table 2). Potatoes were inoculated for 5 d with the weakly virulent isolate Vn-1 at a spore concentration of 1×10^6 conidia mL^{-1}, followed by the highly virulent isolate Vd-36 at a spore concentration of 1×10^7 conidia mL^{-1}. Compared to other treatments, ROS increased at 12 hpi and then gradually decreased (Figure 2A), whereas inoculation with Vn-1 alone resulted in a significant burst of ROS at 24 hpi, compared to inoculation with Vd-36 alone (Figure 2B,C). The changes in H_2O_2 levels were consistent with those in ROS levels (Figure 3A). No changes were observed in the control group (Figure 2D).

Table 2. Four different experimental groups.

Treatments	Inoculation Mode	Inoculation Concentration	Inoculation Dosage
Vn-1 + Vd-36	Potatoes inoculated with Vn-1 for 5 d and then challenged with Vd-36	Vn-1: 1×10^6 conidia mL^{-1} Vd-36: 1×10^7 conidia mL^{-1}	20 mL 20 mL
Vn-1	Potatoes inoculated with Vn-1 alone	1×10^7 conidia mL^{-1}	20 mL
Vd-36	Potatoes inoculated with Vd-36 alone	1×10^7 conidia mL^{-1}	20 mL
CK	Potatoes inoculated with sterile water		20 mL

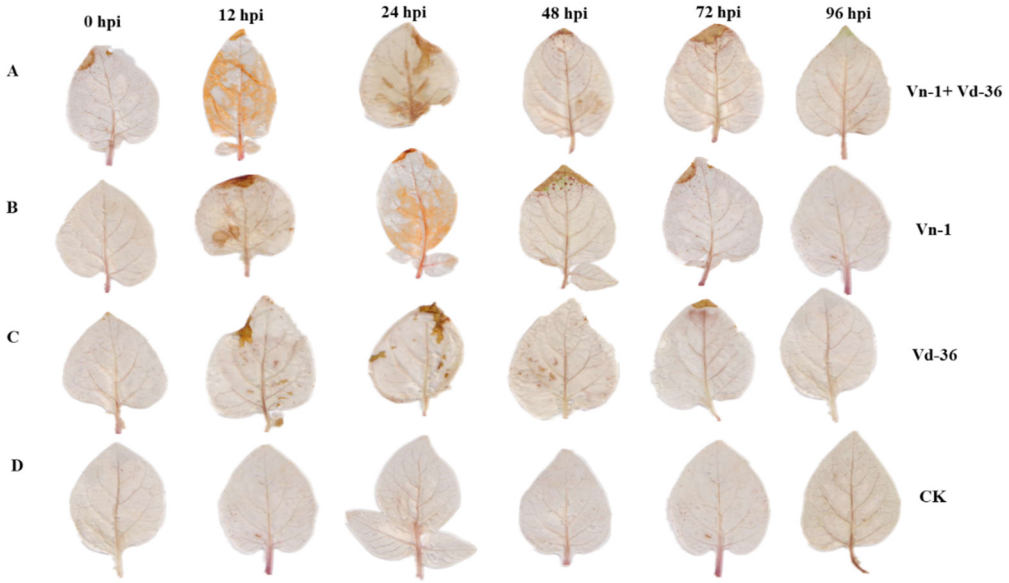

Figure 2. Reactive oxygen species (ROS) burst in different treatments as assessed by 3,3′-diaminobenzidine (DAB) staining: (**A**) potatoes inoculated with Vn-1, followed by Vd-36 inoculation after 5 d; (**B**,**C**) potatoes inoculated with Vn-1 and Vd-36 alone, respectively; (**D**) sterile water was used to inoculate the controls.

3.4. H_2O_2 Accumulation and Related Enzyme Activities

CAT, POD, SOD, and PAL are widely expressed in microorganisms. These enzymes play an important role in the removal of ROS and in defense against pathogen infection [34]. Our experimental results showed that under the treatment of Vn-1 and Vd-36, the content of these defense enzymes, except PAL, increased rapidly at 12 hpi, compared with other time points, began to decline after 24 hpi, and then gradually plateaued. When the isolates Vn-1 and Vd-36 were used individually to inoculate potatoes, the content of defense enzymes, apart from PAL, were the highest at 24 hpi, and the levels were markedly higher when Vn-1 alone was used to inoculate potatoes than when Vd-36 alone was used. This indicated that the attenuated isolate Vn-1 could induce *Verticillium* wilt resistance in potatoes, and at 24 hpi, an ROS burst, the highest content of H_2O_2, and the highest content of three defense enzymes (barring PAL) were observed in potatoes. In view of the above experimental results, we speculated that when the attenuated isolate was used to initially inoculate plants and thereafter the strongly virulent isolate was used, the plants may produce signal responses to the attack of the strongly virulent isolate in advance and stimulate a series of physiological and biochemical reactions against the invasion of pathogens.

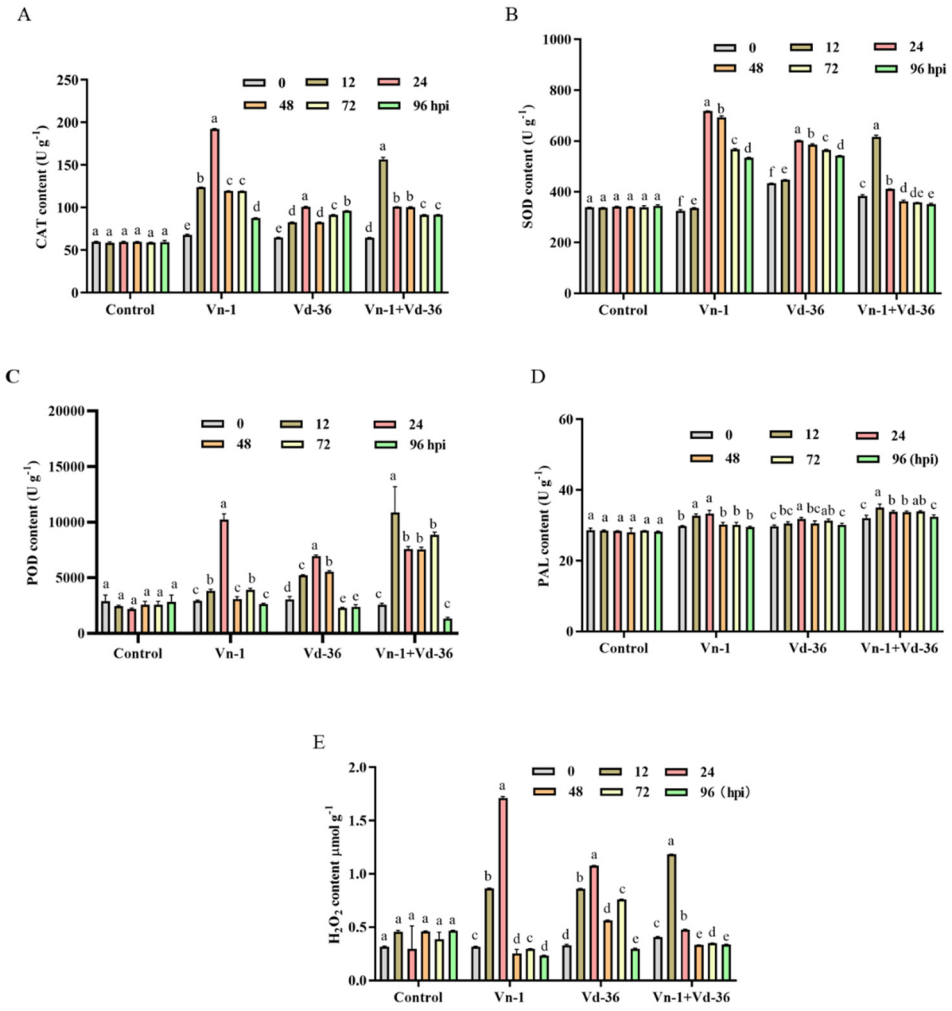

Figure 3. H_2O_2 accumulation and related defense enzyme contents of potato leaves in different treatment groups: (**A**–**D**) were catalase (CAT), peroxidase (POD), distance (SOD), and phenylalanine ammonia-lyase (PAL) activities, respectively; (**E**) is the accumulation of H_2O_2. The letters above represent significant differences (Tukey's multiple comparisons, $p < 0.05$).

3.5. Attenuated Vn-1 Mainly Protects Potatoes from Vd-36 Attack by Activating SA Signalling Pathway

Xue et al. reported that silicon induces resistance to late blight in potatoes by activating the JA/ET signalling pathway and inhibiting the SA signalling pathway [17]. In our study, to determine the changes of signal hormones in potatoes after inoculation with attenuated isolate Vn-1 and then strongly virulent isolate Vd-36, we measured the SA and JA/ET content and the relative expression of related signal pathway genes in potato leaves. As shown in Figure 4A, for the treatment (Vn-1 + Vd-36), the SA content in potatoes were higher at 0, 12, and 24 hpi than that in the control group, except at 48 hpi. The highest level was reached at 12 hpi, and it gradually decreased after 24 h, which was consistent with the changes in the activities of ROS, H_2O_2, and related defense enzymes. The relative expression of StNPR1 [35], a key regulator of SA signalling, was significantly upregulated at 12 and 24 hpi, but a distinct increase was observed at 12 hpi (Figure 4B). These results suggest that SA biosynthesis and signalling are enhanced in potatoes upon invasion by pathogenic bacteria and that the expression of disease resistance genes is activated, resulting in improved disease resistance in potatoes.

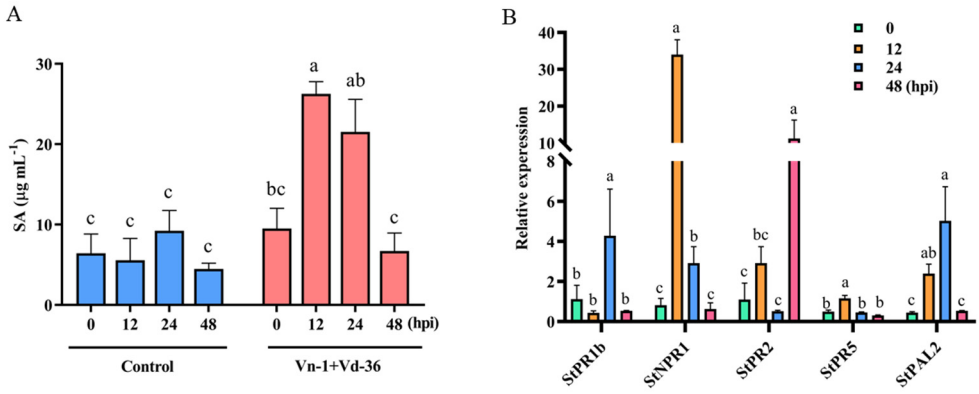

Figure 4. Vn-1 triggers defense *PRs* genes to improve jasmonic acid (JA) production in potatoes: (**A**) salicylic acid (SA) content at different times. Three leaves were collected from each plant, and each treatment consisted of three plants and was repeated three times. Values are mean ± SE (n = 3); sterile water was used as control; (**B**) the relative expression of related defense genes in SA signalling pathway was measured by real-time polymerase chain reaction; letters above bars indicate the significant differences between groups (Tukey's multiple comparisons, $p < 0.05$). Three biological replicates were used.

3.6. JA/ET Signalling Pathways Are Activated Weakly by Attenuated Vn-1

The levels of JA/ET and several synthesis-related genes were measured, and no significant differences were observed between the treatment (Vn-1 + Vd-36) and control groups (Figure 5A). The relative expression of the JA synthesis-related genes *StOPR3* and *StLOX* was higher at 12 hpi, which was consistent with that of the ET synthesis-related genes *ACS1* and *ACS7* (Figure 5B). However, changes in JA/ET-related genes at 12 hpi and other key inoculation time points were considerably weaker than those of SA (Figure 4B). This indicates that Vn-1 activates both the SA and JA/ET signalling pathways, thereby protecting potatoes from the highly virulent isolate Vd-36.

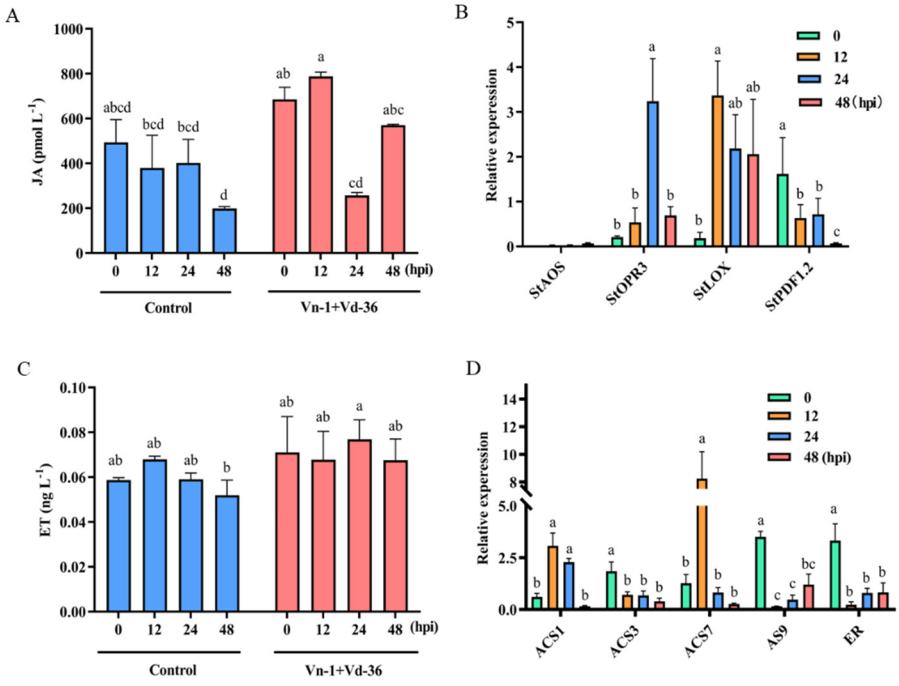

Figure 5. Jasmonic acid (JA)/ethylene (ET) signalling pathways were weakly activated by Vn-1 in potatoes: (**A**,**C**) salicylic acid (SA) content at different times during the control and Vn-1 + Vd-36 treatments.

The experiment was setup in three replicates with three potato plants per replicate, and three leaves per potato plant were collected. Values are shown as means ± SE (n = 3); sterile water was used as a control; (**B**,**D**) relative expression of synthesis genes in JA/ET signalling pathways; letters above bars indicate significant differences between treatments (Tukey's multiple comparisons, $p < 0.05$). Three biological replicates were conducted.

4. Discussion

Potatoes are an important food crop worldwide [1]. *Verticillium* wilt caused by *V. dahliae* is among the most destructive diseases that limit potato production [2]. The less virulent *V. dahliae* isolate Vd171 and *G. nigrescens* CVn-WHg-induced cotton against *Verticillium* wilt [16]. Furthermore, the attenuated isolates *Rhizoctonia solani* BS-J-06-8-1, chd-YT-3-5, and DL-YT-06-4-9 triggered activation of resistance genes in rice [36]. In our previous study, the sunflower was inoculated with the attenuated isolate Vn-1 with the concentration 1×10^6 conidia mL^{-1} first and then inoculated with the most virulent isolate *V. dahliae* V33 after an interval of 5 days, which showed the best effect in protecting sunflower from *Verticillium* wilt. Furthermore, RNA-seq sequencing analysis was used to analyze the differential gene expression of sunflower induced by Vn-1 and V33, respectively. The 20 most significant differentially expressed genes were upregulated in roots of sunflower at 24 hpi and 48 hpi, which included some genes related to resistance response, such as MyB4-related protein genes, xyloglucase/hydrolase protein genes, chitinase and transglutaminase, etc. In search of the effective results against *Verticillium* wilt on other crops by Vn-1, we conducted the present study on induced resistance of Vn-1 to *Verticillium* wilt in potatoes by assessing the activity of several enzymes, SA, JA/ET signalling pathways, and relative expression of related defense genes involved in plant defenses. The results showed that Vn-1 induced *Verticillium* wilt resistance in potatoes by activating the SA signalling pathway and related defense genes, but only weakly activated the JA/ET signalling pathways. Our study provides a theoretical basis for exploring novel avenues of biological control of *Verticillium* wilt in potatoes.

Systematic studies on induced disease resistance began in the 1950s, and a series of studies have shown that disease resistance could be significantly improved by stimulated inoculation (induced inoculation) of distal tissues of pretreated plants through SAR generation [37]. The infection index of apple calli treated with *Valsa mali var. mali (Vmm)* LXS081501 followed by LXS080601 was significantly lower than that after inoculation with strongly virulent LXS080601 alone, and defense mechanisms such as PAL, PPO, and POD improved remarkably [38]. The tannin, ferulic acid, and chlorogenic acid content in cucumber leaves peaked on days 11, 7, and 9, respectively, after the seeds and seedlings were inoculated with the attenuated isolate *Fusarium oxysporum f. sp. cucumerinum* (FOC-RA-5), and these three substances were positively correlated with plant disease resistance [39]. Tomatoes can be inoculated against late blight using spore suspensions of six less-virulent isolates of *Phytophthora infestans* [40]. The findings of the present study showed that potatoes exhibited the best resistance to *Verticillium* wilt after inoculation with the weakly virulent isolate Vn-1 in the conidial suspension (1×10^6 conidia mL^{-1}), followed by inoculation with the most virulent isolate Vd-36 at an interval of 5 d. Moreover, ROS and H_2O_2 reached their highest levels in potatoes at 12 hpi, in line with the levels of defense enzymes, such as CAT, SOD, and POD.

The PTI and ETI immune pathways are induced when plants are attacked by pathogens, which leads to SA accumulation. PTI indirectly activates EDS1 and PAD4 and further promotes the expression of PAL and ICS, leading to progressive accumulation of SA [38]. However, in the ETI response, the R genes in host cells are recognized by effector proteins secreted by the pathogen, which activate signalling pathways downstream of SA [20]. NPR1 as an important factor is the main regulator of the SA signalling pathway. When the concentration of SA increased, the NPR1 monomer was increased. Important factors, such as TGA and WRKY, were activated, ultimately leading to the upregulation of related defense genes (e.g., *PR1*, *PR3*, *PR5*) [41–43]. A previous study elucidated the signalling

mechanism of host–pathogen interactions by comparing the differences in the expression of defense genes of SA-related signalling pathways in plants between the less-virulent isolate vs. 06-14 and the strongly virulent isolate Vd 1396-9. Furthermore, spraying *Bacillus amyloliticus* MBI600 protects potatoes against potato virus Y infection and activates the tomato SA signalling pathway [44].

The amount of SA in potatoes was higher than that in the control group at 0, 12, and 24 hpi, except at 48 hpi. It was the highest at 12 hpi but declined after 24 hpi, together with the upregulation of the genes *StNPR1*, *StPR2*, *StPR5*, and *StPAL2*. The relative expression of *StPR1b* was also upregulated at 24 hpi. The results of our study showed that SA biosynthesis and signal transduction pathways were enhanced and related defense genes were activated by the less-virulent isolate Vn-1 to avoid attack by the virulent isolate Vd-36 (Figure 4).

SA and JA/ET are important for defense against pathogens. SA typically mediates the ability of plants to withstand biotrophic pathogens, whereas JA/ET typically acts against necrotrophic pathogens. In the mediated process, the three approaches are independent and intersected, depending on the interconnection between the pathogen and host plant. Foliar application of Si enhances LB resistance through JA-, ET-, and NPR1-independent signalling pathways [17]. Silicon protects plants from *Phytophthora sojae* by mediating SA and JA signalling pathways [45]. However, no significant difference in JA/ET content between the control and treated (Vn-1 + Vd-36) potatoes was observed when the attenuated isolate Vn-1 was applied. Additionally, the expression of JA/ET synthesis-related genes was lower than that of SA at all other time points. In general, in our study, the weakly virulent strain of potato Vn-1 treatment provided strong protection against the highly virulent strain Vd-36, mainly by mediating the SA but not the JA/ET weak signalling pathway, in contrast to the findings of some previous studies (Figure 6).

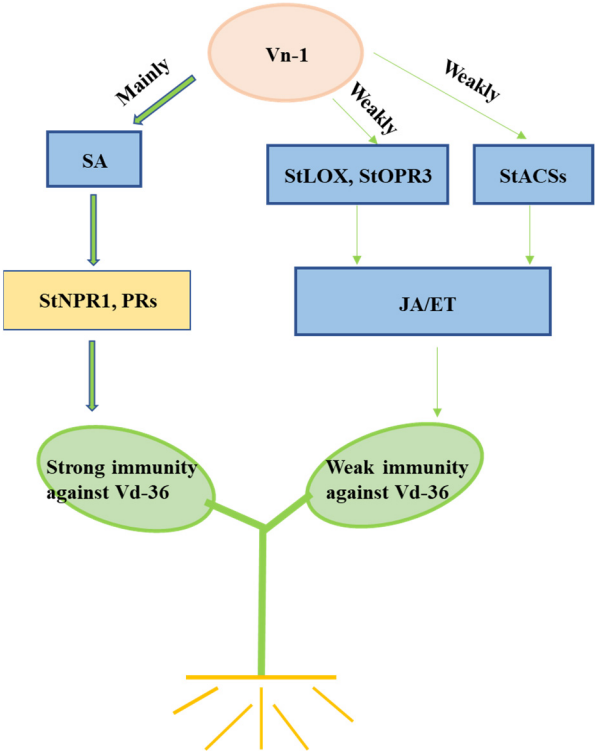

Figure 6. Network diagram of predicted Vn-1-induced resistance of potatoes against *Verticillium dahl iae.* Inoculation treatment with Vn-1 in advance mainly stimulated salicylic acid (SA) accumulation and biosynthesis-related genes (*StNPR1*, *PRs*), and only slightly stimulated jasmonic acid (JA)/ethylene (ET) accumulation and related genes (*StLOX*, *StOPR3*/*StACSs*) during induction of resistance to Vd-36 in potatoes.

5. Conclusions

The long-term use of chemical pesticides to control plant diseases has resulted in a series of problems, such as emerging pathogen resistance and pesticide residues, which severely threaten the environment and preclude safe use of such products in agriculture. Therefore, there is an urgent need to develop economical and efficient methods and technologies for plant protection [46,47]. This study on induced resistance of potatoes to *Verticillium* wilt through the less virulent isolate Vn-1 provides theoretical guidance for further exploration of a new method of biological control of *Verticillium* wilt in potatoes, such as the development and application of plant vaccines. Zheng et al. (2013, 2017) isolated a weak wild attenuated strain, *Ralstonia solanacearum* FJAT-1458 and found that it can effectively induce tomato resistance to bacterial wilt. Furthermore, the preparation technology of the plant vaccine agent against bacterial wilt in tomatoes was advanced, and the control effect reached 100% in laboratory experiments [48,49]. Therefore, it is essential to identify substances that act against pathogens. Our results may thus contribute to the research and development of biopesticides and help promote sustainable development of agriculture and protect the ecological environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123082/s1, Table S1: Details of 72 isolates; Table S2: Primers used in the study.

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