



INVESTIGATION OF THE EFFECTS OF SOME PLANT ACTIVATORS AGAINST VERTICILLIUM WILT (*Verticillium dahliae* Kleb.) ON COTTON

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Abstract: Wilt disease caused by *Verticillium dahliae* Kleb. is one of the stress factors affecting yield and fiber quality traits in cotton cultivation. Plant activators provide resistance by stimulating genes that activate the resistance mechanism in the plant. The aim of this study is to determine the effect of plant activators such as auxiGRO, Green Miracle, Maxicrop, ProAct Plus, and Sojall Vitanal against *Verticillium* wilt under both *in vitro* and *in vivo* conditions. Firstly, the effect of various concentrations of plant activators (0, 1, 5, 25, 100, 250, and 500 ppm) on mycelial growth of two fungal isolates of *V. dahliae* (PHCVd3-non-defoliating pathotype and PHCVd47-defoliating pathotype) in potato dextrose agar (PDA) media was investigated *in vitro*. The effect of plant activators on *V. dahliae* was determined in tolerant cotton plants (cv Carmen) and susceptible cotton plants (cv Acala SJ2) in two different ways seed coating and foliar application *in vivo*. *In vitro* experiments were carried out with three replicates, and *in vivo* experiments were with five replicates depending on a completely randomized plot design. 500 ppm dose of all plant activators inhibited the mycelial growth of both isolates by approximately 90%. The lowest disease index (DI) against PHCVd3 was determined as 1.43 in the tolerant cv Carmen with seed coating of auxiGRO. The lowest DI against PHCVd47 was found in Sojall Vitanal and ProAct Plus at 2.09 and 2.12, respectively. The lowest DI against both isolates was found as 1.42 and 2.18 in cv Carmen by foliar application of ProAct Plus, respectively. Plant activators did not show any inhibitory effect on disease severity against both isolates in cv Acala SJ2. The combination of tolerant cultivar + plant activators can be suggested against *Verticillium* wilt disease as an alternative control.

Keywords: *Verticillium dahliae*, Plant activators, Disease index, Alternative control, Cotton

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1. Introduction

Cotton is a leading source of natural fiber, cotton seed oil, and livestock feed in more than 80 countries, covering an area of ~35 million hectares (Jans et al., 2021). Upland cotton (*Gossypium hirsutum* L.) ranks first in world production and accounts for 90% of world cotton production (Chen et al., 2007). Therefore, 99.5% of Türkiye's cotton is *G. hirsutum* L. species cotton (Gürel et al., 2000). Türkiye is the sixth largest cotton producer in the world after China, India, USA, Brazil, and Pakistan (USDA, 2021). In Türkiye, cotton is grown in 477.000 hectares in 4 main regions, yielding 2.2 million tons of seed cotton yield (TSI, 2021).

The soil-borne fungus *Verticillium dahliae* causes *Verticillium* wilt, one of the most important diseases of cotton (Klosterman et al., 2009). The fungus infects more than 400 plant species, including cotton (Berlanger and Powelson, 2000). *Verticillium* wilt results in a yield loss of around 10-35% worldwide (Song et al., 2020).

Nowadays, places where cotton is grown have both defoliating and non-defoliating pathotypes of the disease (Bejarano-Alcazar et al., 1995). The fungus survives in the soil as microsclerotia. Microsclerotia form in the dead plant, thus increasing the inoculum potential for future years (Huisman and Ashworth, 1976). Pathogen blocks the movement of water and other minerals from the root to the leaves and tissues. Then it causes wilting, desiccation, reduced photosynthesis, shedding of small bolls, and changes in yield and fiber quality characteristics, starting with the lower leaves (Agrios, 2005). Today, an effective chemical control against *Verticillium* wilt has not been developed yet. In this context, alternative control methods are needed in the control against *V. dahliae*.

The use of plant activators for sustainable agriculture is increasing day by day. The development and practical use of new substances that stimulate the physiological activity of plants have gained momentum (Akbudak and Tezcan, 2006). These substances are a source of plant



growth regulators, organic osmolytes, amino acids, and macro and micronutrients (Khan et al., 2009). Plant activators are environmentally friendly and non-toxic preparations that increase plant growth by positively affecting the morphological and physiological properties of plants (Kunicki et al., 2010), strengthen the natural defense mechanisms of plants, and increase the resistance of plants to abiotic and biotic stress factors (Bulgari et al., 2015). Hcm1 containing harpin protein suppressed the growth of *V. dahliae* and *Fusarium oxysporum*, at the same time Hcm1 can activate innate immunity and prevent *Verticillium* and *Fusarium* wilt in cotton (Zhang et al., 2016). Green Miracle plant activator is a long-chain fatty acid-based new-generation stress alleviator for improving the plant health (Bursalioglu and Aki, 2018). The study aims to determine the effect of some plant activators against two fungal isolates of *V. dahliae* (PHCVd3-non-defoliating pathotype and PHCVd47-defoliating pathotype) under both *in vitro* and *in vivo* conditions.

2. Materials and Methods

2.1. Plant Materials, Plant Activators and Fungal Pathogen

Cotton cultivars (cv) tolerant Carmen (*Gossypium hirsutum* L.), and susceptible Acala SJ2 (*G. hirsutum* L.) were used as plant material (Bolek et al., 2005; Erdoğan et al., 2014). Plant activators such as auxiGRO (30 g/100 l water), Green Miracle (200 ml/100 l water), Maxicrop (30 g/100 l water), ProAct Plus (10 g/100 l water), Sojall Vitanal (60 ml/100 l water) licensed in many cultivated plants including cotton were used in the study. Pure cultures of *V. dahliae* (PHCVd3 isolate-non-defoliating pathotype; PHCVd47 isolate-defoliating pathotype) were provided by Prof. Dr. Şener KURT (Hatay Mustafa Kemal University/Türkiye). Pathogen isolates were grown in the dark at 24±1°C for 14 days and after that subcultured on potato dextrose agar (39 g/l, PDA-Difco) media.

2.2. In-Vitro Studies

2.2.1. Determination of the effects of plant activators on mycelial growth of isolates of *V. dahliae*

A variable concentration of plant activators (1, 5, 25, 100, 250, and 500 ppm) was added to the sterilized PDA medium before being dispensed in 25 ml portions into sterilized Petri plates (90 mm). PDA medium with plant activators was kept at room temperature for 24 hours. A single 5-diameter- mycelium disc taken from the leading growth edge of 7-day-old cultures of both pathotypes of *V. dahliae* grown on PDA was placed in the center of a Petri dish containing plant activator + PDA. As a control, a disc of *V. dahliae* was grown on a PDA plate. The radius of each fungal colony was measured after a 14-day incubation at 24±1 °C in the dark. The relative growth inhibition was expressed as a percentage [(control-treatment)/control x 100] (Deans and Svoboda, 1990). This experiment was performed using a fully randomized parcel design with three replicates and was replicated twice.

2.3. In Vivo Trials

2.3.1. Determination of the effects of seed coating applications of plant activators against *V. dahliae*

The effects of five plant activators against *Verticillium* wilt were tested in a plant growth room on two cultivars of cotton plants (cvs Carmen and Acala SJ2). The coated with plant activators and control seeds were each planted into 10-cm diameter plastic pots containing an autoclaved soil-sand-peat (1:1:1) mixture. Then, when the cotton seedlings reached the cotyledon stage, thinning was performed, and one seedling was left in each pot. To determine the susceptibility of plant activators-coated cotton cultivars to *V. dahliae* (Erdoğan et al., 2014) two-week-old spores cultured in broth medium (0.01 g FeSO₄·7H₂O, 0.5 g MgSO₄·7H₂O, 2 g NaNO₃, 1 g K₂HPO₄, 0.5 g KCl, and 7.5 g sucrose, 1 l sterile distilled water) cultured isolates of PHCVd3 and PHCVd47 were filtered through 2 layers of cheesecloth and mycelium and pieces of agar were removed from the suspension and then the spore concentration was adjusted to 4 × 10⁶ spores/ml using a Thoma slide in the light microscope (Leica) and used for the inoculation of cotton plants. The plants were transplanted into new plastic pots with spore solution (10 ml) when they reached the six-true-leaf stage. Plants were incubated at 24±1°C, with 12 hours light / 12 hours dark conditions. Control plants were applied with sterile water. 30-35 days after inoculation, disease severity was assessed for each plant on a 0-to-5 rating scale according to the percentage of foliage affected by acropetal chlorosis, necrosis, wilt, and/or defoliation (0 = no symptoms, 1 = chlorosis in the lower leaves, 2 = moderate (30-50% of the leaves) wilt with severe chlorosis, 3 = moderate wilting and necrosis, 4 = severe (more than 50% of leaves) wilting and necrosis, 5 = dead plant) (Tsrör et al., 2001). The pot experiment was performed with five replicates in a fully randomized parcel design.

2.3.2. Determination of the effects of foliar applications of plant activators against *V. dahliae*

Plants of Carmen and Acala SJ2 were grown in the plant growth room until the six-true-leaf stage. As in the seed coating application, *V. dahliae* isolates were obtained and spore concentrations were adjusted. Pathogen isolates were applied according to the conidia suspension technique. Recommended doses of plant activators were sprayed on the leaves of cotton plants as a first application one day after pathogen application. The second application was sprayed 7 days after the first application and the third application was sprayed 14 days later. Control plants were sprayed with sterile water. 30-35 days after inoculation, disease severity was assessed for each plant on a 0-to-5 rating scale. The experiment was performed with five replicates in a fully randomized parcel design. The disease index (DI) value was determined according to the method described by Karman's (1971) formula (Equation 1)

$$DI = (ax0) + (bx1) + (cx2) + (dx3) + (ex4) + (fx5)/M \quad (1)$$

where a, b, c, d, e and f are the plant numbers with degrees 0, 1, 2, 3, 4, and 5, respectively, and M is the overall plant number.

2.4. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) using JMP software version 13 (SAS Institute Inc., Cary, NC, USA). Statistical software and the means were grouped using the LSD (0.01) test (Genç and Soysal, 2018).

3. Results and Discussion

3.1. The Effects of Plant Activators on Mycelial Growth of Isolates of *V. dahliae*

The plant activator doses were found to be significant according to the statistical analysis results ($P \leq 0.01$) of the *in vitro* experiment. In the high dose (500 ppm) application of Maxicrop, the lowest mycelial diameter (MD) was found at 1.00 mm and 2.83 mm, respectively; the highest effect was determined at 96.80%, and 91.00% respectively, against PHCVd3 and PHCVd47 isolates. In the high dose of Sojall Vitanal, the lowest MD was found at 1.00 mm and 3.83 mm against PHCVd3 and PHCVd47 isolates, respectively; the highest effect was detected at 96.70% against PHCVd3 and 87.80% against PHCVd47, respectively. In the high dose of Green Miracle, the lowest MD was determined at 1.08 mm and 3.42 mm, respectively; the highest inhibition rate was measured at 96.50% and 89.20%, respectively against both isolates. In the high dose of ProAct Plus, the lowest MD was found at 1.08 mm and 4.00 mm, respectively; the highest effect was determined at 96.50% and 87.30%, respectively against both isolates. In the high dose of auxigRO, the lowest MD was found at 1.42 mm and 4.58 mm, respectively, and the highest effect was found at 95.60% and 85.50%, respectively against both isolates (Table 1).

In a similar study, Yildirim and Yapici (2007) reported that the harpin protein was 76% effective at 1000 µg/ml concentration and showed a high effect even at low doses against the gray mold caused by *Botrytis cinerea* in strawberries. Zhang et al. (2016) stated that Hcm1-containing harpin protein suppresses the growth of *V. dahliae* and *Fusarium oxysporum in vitro*, and that Hcm1 can activate innate immunity. Aysan et al. (2019) have reported that salicylic acid (SA) and Fosetyl-Al at concentrations above 700 µg/ml *in vitro* suppressed mycelial growth of strawberry black root rot caused by *R. solani*, plant activators such as Acibenzolar-S-Methyl (A-S-M), Messenger, ISR-2000, Crop-Set did not inhibit

mycelial growth of the pathogen.

Our results are not similar to Şahbaz and Akgül (2016) found that the plant activators Fosetyl-al, SA, A-S-M + Metalaxyl-m and ISR-2000 did not inhibition against mycelial growth of *F. oxysporum* f. sp. *vasinfectum* and *V. dahliae in vitro*.

3.2. The Effects of Seed Coating Applications of Plant Activators on *V. dahliae*

Application x cultivar interaction was found to be significant according to the statistical analysis results ($P \leq 0.01$) of the *in vivo* experiment. Disease index (DI) values of PHCVd47 isolate were higher than PHCVd3 isolate in tolerant cv Carmen and susceptible cv Acala SJ2. The lowest DI value against the PHCVd3 isolate was obtained from the plant activator of auxigRO (1.43) in the tolerant cotton plant (cv Carmen) (Figure 1), followed by the plant activator of Green Miracle (1.50) and Sojall Vitanal (1.54). The lowest DI value against the PHCVd3 isolate was found in the plant activator Maxicrop (2.92) in the susceptible cotton plant (cv Acala SJ2). The lowest DI value against the PHCVd47 isolate was obtained from the plant activators of Sojall Vitanal (2.09) and ProAct Plus (2.12) in cv Carmen (Figure 1), and these activators were statistically in the same group. The lowest DI value was found in the plant activator Maxicrop (3.28) in cv Acala SJ2 (Table 2).

According to our results, plant activators containing GABA + L-Glutamic acid, *Lactobacillus acidophilus*, and harpin protein active substances showed the highest effect against both pathotypes of the pathogen. Harpin protein activates the natural resistance mechanism of plants against diseases and pests and increases the resistance of the plant against pathogen attack (Strobel et al., 1996). Kinnersley and Turani (2000) reported that GABA increased the plant's resistance to disease as it reduced stress against the pathogen. *L. acidophilus* has been reported to increase plant resistance through induced systemic resistance (Anonymous, 2017). Our results are in agreement with those of Tosun et al. (2003) reported that applied SA and harpin alone and together with fungicide (Agrifos 400), then infected the plants with *Phytophthora infestans* and found SA 47%, harpin 55%, Agrifos 400 88% suppressed the disease. High efficacy results have been obtained in Messenger Gold (MG), MG + ERS-T22 Planter Box + Bordeaux mixture, and MG + Bordeaux mixture applications. Application of MG and in combination with MG suppressed the *Verticillium* wilt disease (Arici and Demirtas, 2019).

Table 1. Inhibitory effect of plant activators on mycelial growth of isolates of *V. dahliae*

Plant activators	Dose (ppm)	PHCVd3 isolate		PHCVd47 isolate	
		MG (mm)	MGI	MG (mm)	MGI
auxiGRO	1	23.75 b*	25.40	28.25 b	10.80
	5	19.67 c	38.20	23.75 c	25.00
	25	14.75 d	53.70	19.83 d	37.40
	100	10.17 e	68.10	14.83 e	53.20
	250	5.50 f	82.70	9.75 f	69.20
	500	1.42 g	95.60	4.58 g	85.50
	Control	31.83 a	0.00	31.67 a	0.00
F _{application}			**		**
CD _(p=0.01)			6.6		3.3
Green Miracle	1	25.50 b	21.40	27.33 b	13.70
	5	17.58 c	43.50	19.58 c	38.20
	25	13.92 d	55.40	18.50 c	41.60
	100	8.75 e	71.90	11.75 d	62.90
	250	4.42 f	85.80	7.33 e	76.80
	500	1.08 g	96.50	3.42 f	89.20
	Control	31.17 a	0.00	31.50 a	0.00
F _{application}			**		**
CD _(p=0.01)			6.7		3.9
Maxicrop	1	24.08 b	22.80	26.00 b	17.00
	5	16.08 c	48.50	21.33 c	31.70
	25	11.08 d	64.50	13.00 d	58.30
	100	6.92 e	77.90	8.83 e	71.70
	250	4.00 f	87.20	5.33 f	82.90
	500	1.00 g	96.80	2.83 g	91.00
	Control	31.17 a	0.00	31.17 a	0.00
F _{application}			**		**
CD _(p=0.01)			9.7		3.6
ProAct Plus	1	23.92 b	23.10	27.58 b	12.10
	5	15.67 c	49.60	23.42 c	25.70
	25	12.08 d	61.10	18.67 d	40.60
	100	9.33 e	70.00	13.58 e	56.80
	250	5.00 f	83.90	7.17 f	77.10
	500	1.08 g	96.50	4.00 g	87.30
	Control	31.08 a	0.00	31.42 a	0.00
F _{application}			**		**
CD _(p=0.01)			8.1		3.3
Sojall Vitanal	1	23.58 b	22.50	27.42 b	12.10
	5	17.08 c	43.80	22.17 c	28.90
	25	11.50 d	62.20	17.92 d	41.90
	100	8.50 e	72.00	14.08 e	54.70
	250	3.50 f	88.50	8.58 f	72.50
	500	1.00 g	96.70	3.83 g	87.80
	Control	30.42 a	0.00	31.17 a	0.00
F _{application}			**		**
CD _(p=0.01)			6,9		2.6

*Mean values followed by different letters within the column are significantly different according to LSD Test, **($P \leq 0.01$), MG= mycelial growth, MGI= mycelial growth inhibition (%), CD= critical difference.

3.3. The Effects of Foliar Applications of Plant Activators on *V. dahliae*

The statistical analysis of the pot experiments data revealed that application x cultivar interaction differences were significant ($P \leq 0.01$). DI values of PHCVd3 and PHCVd47 isolates were lower in tolerant cotton plants (cv Carmen) than susceptible cotton plants

(cv Acala SJ2). The lowest DI value against PHCVd3 isolate was found in the application of ProAct Plus (1.42) in tolerant cotton plants (cv Carmen) (Figure 2). The lowest DI value was determined in susceptible cotton plants (cv Acala SJ2) with Maxicrop (3.02) and Green Miracle (3.07). The lowest DI value against PHCVd47 isolate was detected in the application of ProAct Plus

(2.18) in tolerant cv Carmen (Figure 2). The lowest DI value was found in susceptible cv Acala SJ2 with Maxicrop (3.29) (Table 3).

The highest effect of plant activators sprayed on leaves was again determined in plant activators with GABA + L-Glutamic acid, *L. acidophilus*, and harpin protein active ingredient. In a similar study, Bishnoi and Pavyavula (2004) reported that Harpin and A-S-M activators reduced the severity of leaf blight by 8-12% in tomato

cultivars and did not affect scab of canola in canola cultivars. Delisoy and Altınok (2019) determined that auxiGro reduced disease development by 49.25%, Crop-Set by 41.80% and, ISR-2000 by 35.82% compared to positive control. Tuğlu (2019) reported that *L. acidophilus* yeast extract and Benzoic acid, A-S-M + Metalaxyl-M and harpin protein reduced the severity of hazelnut powdery mildew disease by 49.26%, 55.83%, and 33.34%, respectively.

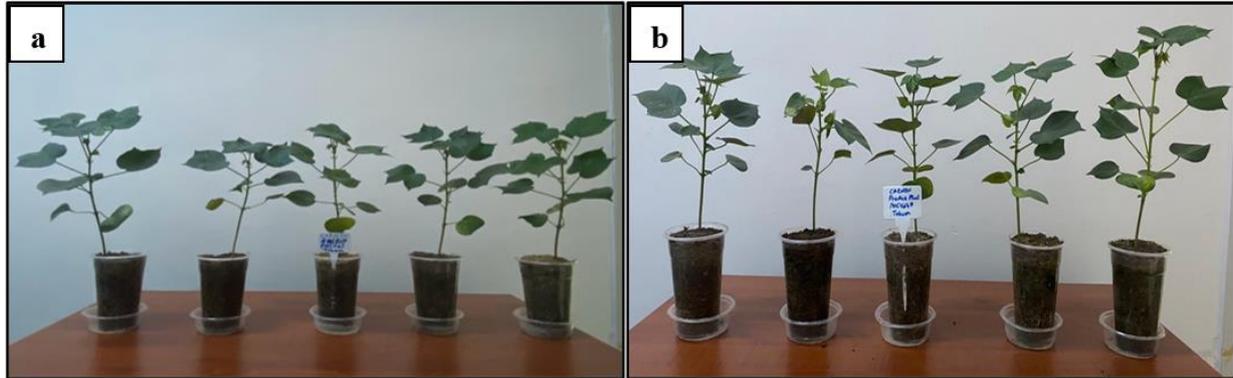


Figure 1. (a) Effect of auxiGRO against PHCVd3 isolate in the seed coating tolerant Carmen cultivar, (b) Effect of ProAct Plus against PHCVd47 isolate in the seed coating tolerant Carmen cultivar.

Table 2. Disease index values in cotton cultivars seed coated with plant activators after PHCVd3 and PHCVd47 inoculation

Plant activators	Carmen cultivar		Acala SJ2 cultivar	
	PHCVd3 DI	PHCVd47 DI	PHCVd3 DI	PHCVd47 DI
auxiGRO	1.43 c*	2.20 ab	3.08 bc	3.54 b
Green Miracle	1.50 bc	2.17 ab	3.00 bc	3.50 bc
Maxicrop	1.59 b	2.21 ab	2.92 c	3.28 c
ProAct Plus	1.58 b	2.12 b	3.20 b	3.60 b
Sojall Vitanal	1.54 bc	2.09 b	3.12 b	3.67 b
Control	1.76 a	2.32 a	3.61 a	4.20 a
F _{cultivar x application}	**	**	**	**
CD(P=0.01)	5.6	5.5	4.9	4.9

*Mean values followed by different letters within the column are significantly different according to LSD Test, **P≤0.01, DI= Diseases index, CD= Critical difference.

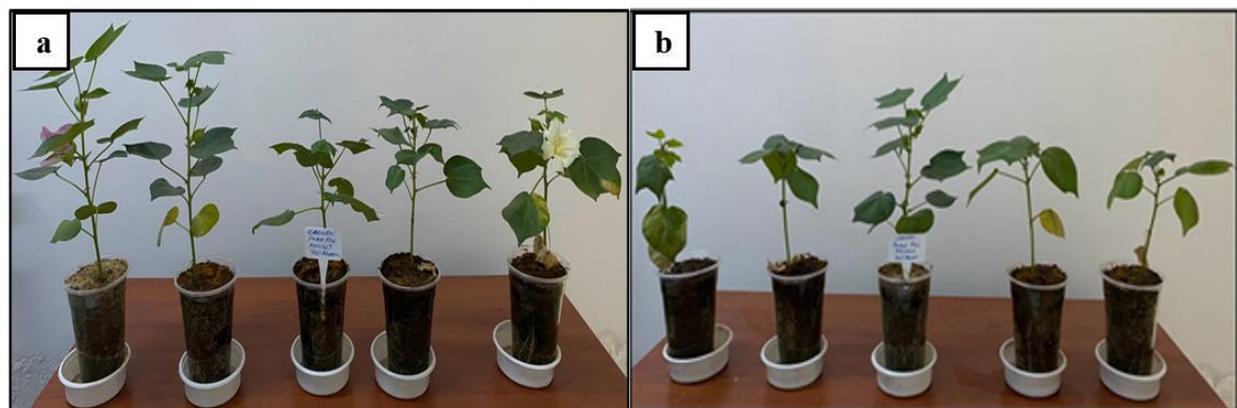


Figure 2. (a) Effect of ProAct Plus against PHCVd3 isolate, (b) PHCVd47 isolate in the foliar application tolerant Carmen cultivar.

Table 3. Disease index values in cotton cultivars foliar applicated with plant activators after PHCVd3 and PHCVd47 inoculation

Plant activators	Carmen cultivar		Acala SJ2 cultivar	
	PHCVd3 DI	PHCVd47 DI	PHCVd3 DI	PHCVd47 DI
auxiGRO	1.50 c*	2.25 b	3.12 cd	3.58 c
Green Miracle	1.57 bc	2.22 bc	3.07 d	3.54 c
Maxicrop	1.64 b	2.25 b	3.02 d	3.29 d
ProAct Plus	1.42 d	2.18 c	3.25 b	3.64 bc
Sojall Vitanal	1.51 c	2.23 bc	3.19 bc	3.72 b
Control	1.80 a	2.35 a	3.66 a	4.35 a
F _{cultivar x application}	**	**	**	**
CD _(P=0.01)	6.7	2.2	2.4	2.4

*Mean values followed by different letters within the column are significantly different according to LSD Test, **P≤0.01, DI= Diseases index, CD= Critical difference.

4. Conclusion

High doses of plant activators MaxiCrop, Sojall Vitanal, ProAct Plus, Green Miracle and auxiGRO inhibited the mycelial growth of isolates of PHCVd3 and PHCVd47 by approximately 90%. AuxiGRO against PHCVd3 isolate, Sojall Vitanal and ProAct Plus against PHCVd47 isolate in tolerant cotton cultivar (Carmen) with seed coating with plant activators, and ProAct Plus plant activator against both isolates in foliar application tolerant cv Carmen was found promising. Plant activators coated on seeds and sprayed on leaves did not reduce disease severity against both pathotypes in susceptible cotton cultivar (Acala SJ2). In this context, the combination of tolerant cultivar + plant activators can be suggested against *Verticillium* wilt disease as an alternative control, which is the best alternative within the scope of integrated control. However, we need detailed studies related to assessing the licensed doses of plant activators in cotton cultivars and *Verticillium* wilt under field conditions.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	Z.S.	O.E.
C	50	50
D	50	50
S		100
DCP	50	50
DAI		100
L	50	50
W		100
CR	50	50
SR		100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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