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THE ELITE USE OF BUTYLATED HYDROXYTOLUENE (BHT) AND SODIUM ALGINATE (SA) FOR THE SUPPRESSION OF *VERTICILLIUM* WILT OF COTTON

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ABSTRACT

Verticillium dahliae Kleb., the cause of *Verticillium* wilt is a major threat, which is known as "cancer" in cotton. However, currently available conventional fungicides are less effective due to its long persistency as mycelia and microsclerotia. The objective of the present study was to find out the optimum and most effective treatment for the suppression of this drastic disease. Ten modern fungicides alone and in combination with butylated hydroxytoluene (BHT) and sodium alginate (SA) were tested under laboratory conditions. The combinations of Carbendazim and Topsin-M with BHT+SA, respectively, decolorized the mycelial plug of *V. dahliae,* indicates their strongest efficacy. Significant improvement in growth parameters of two cotton germplasm Arcot-402bne (High Susceptible) and Yumian-2067 (Tolerant) compared to control-1 (Inoculated and untreated) were obtained under greenhouse conditions with Carbendazim and Topsin-M combinations. These two combinations had the lowest wilt incidence (WI) percentage and showed high efficacy. In contrast, Hymexazol and PCNB combinations and fungicides alone were exhibited lower effectiveness, with the highest WI percentage. No infection (%) of *V. dahliae* was observed with re-isolation for Carbendazim combination, clearly proved the strongest efficacy. The present study determined the new, strongest and optimum chemical combination with a hope will effectively control this serious disease for sustainable cotton production.

INTRODUCTION

Verticillium wilt caused by a fungus, *Verticillium dahliae* Kleb., that inhabits in the soil is considered one of the most devastating and a widespread disease, causes substantial yield losses in cotton worldwide (Bell, 1992). It is also known as "cancer disease" in cotton. This fungus infects the roots of cotton and then gradually grows into the vascular system of the host. Symptoms include marginal chlorosis and/or necrosis in the leaves, wilting, discolouration of vascular bundles in the stem, a decrease in photosynthesis, and an increase in respiration (Pegg, 1989; Hampton *et al.,* 1990). Yield losses due to *Verticillium* wilt vary greatly from one region to another because of differences in the severity of the disease, agro-climatic conditions and pathogenesis.

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The existence of defoliating and non-defoliating strains of *V. dahliae* is also complicated. The defoliating strains are the most virulent, which can cause complete defoliation of severely infected plants that ultimately causes a significant reduction of plant biomass and heavy loss of yield (Bell, 1992; Paplomatas *et al.,* 1992). A significant reduction in seed cotton yield, fibre length, and fibre strength has also been reported (Erdogan *et al.,* 2006). Numerous management practices have been applied to prevent and/or reduce the loss of crops caused by *Verticillium* wilt. Examples of commonly used management practices for *Verticillium* wilt includes cultural practices, soil solarisation (Melero-Vara *et al.,* 1995), organic amendments (Huang *et al.,* 2006) and fungicides (Niu *et al.,* 2006). However, while these practices might suppress the disease to some extent, they could not completely control its severity. Hence, alternative biocontrol agents are also being developed (Huang *et al.,* 2006; Zheng *et al.,* 2011). However, the practical application of these bio-agents remains inadequate. Moreover, few resistant germplasms that are able to overcome this detrimental disease have been successfully screened (Khaskheli *et al.,* 2013). In addition, chemical treatments of soil are also believed to be effective means of controlling *Verticillium* wilt (Rekanovic *et al.,* 2007). The soil fumigation with methyl-bromide has been largely used for the control of this disease (Watson, 1992; Subbarao, 2002), however, because of its detrimental impact on the human health and environment, its use is to be phased out by 2015 (Watson, 1992). Moreover, the use of conventional fungicides (Talboys, 1984; Tian *et al.,* 1998) are also less effective due to survival and capability of fungus in the soil for long time, as mycelia and microsclerotia and quick dispersal through various means of agricultural practices (Rekanovic *et al.,* 2007). Currently no any effective strategy is available to control this serious disease. Consequently, it becomes a challenge for producers to find an effective means of control (Junli *et al.,* 2006).

The *Verticillium dahliae* Kleb usually excreted phytotoxins with oxidation matter which let to develop various symptoms in the cotton plant includes wilt (Keen *et al.,* 1972; Fradin and Thomma, 2006). To prevent from the oxidation of the infected plant with the poison produced by *V. dahlia*, we try to find some antioxidants and combined with conventional fungicides to control the *Verticillium* wilt. Butylated hydroxytoluene (BHT) is one kind of better antioxidants with phenolic compounds since it is safely often added to foods to preserve fats or oils (Babu and Wu, 2008). Oxygen reacts preferentially with BHT rather than plant components, thereby protecting the plant from spoilage. Sodium alginate (SA) is the sodium salt of alginic acid which absorbs water quickly and usually used in thickening drinks, ice cream and cosmetics. In addition, BHT is fat-soluble, thus, SA together with BHT can be used as stabilizer, emulsifier and gelling agent. These compounds can make the fungicides mixes more easily, ultimately can suppress the intensity of *V. dahliae*. Hence, in the current study, we aimed to incorporate SA and BHT with conventional fungicides, which have not been used before, to find out the strongest chemical combinations against *V. dahliae* Kleb. to address the scarcity of effective treatment that is available for the end-user (i.e. crop farmers and managers).

MATERIALS AND METHODS

To realise the objectives of the present study, *in vitro* and greenhouse experiments were conducted twice during two successive years, 2010–2011. The virulent strain D07073-3 of *V. dahliae* and the plant materials includes Arcot-402bne (High susceptible) and Yumian-2067 (Tolerant) germplasm were used in both experiments.

Fungicides and chemical compound formulation

Ten different modern fungicides included Carbendazim (Ca), Topsin-M (TM), Triadimefon or Bayleton (Tri), Thiram (Thi), Quadris-Azoxystrobin (Qu), Iprodione (Ip), Hymexazol (Hy), Huangkujinfen (Hu), Bravo-Chlorothaloni (Br) and PCNB-Pentachloronitrobenzene (PCNB) and two chemical compound included Sodium alginate (SA) and Butylated hydroxytoluene (BHT) were applied for *in vitro* and greenhouse experiments (Table 1). All the fungicides were used alone and in combinations with SA and BHT, however, combinations with SA and BHT are denoted as (+).

In vitro test

The efficacy of 10 different modern fungicides alone and in combination (+) with chemical compound (BHT and SA) was tested under laboratory conditions to reduce *V.dahliae* growth by poison food techniques (Grover and Moore, 1962; Nene and Thapliyal, 1973; Mishra and Tiwari, 1992). The experiment was conducted twice in randomized complete design (RCD) with six replications and three concentrations (1, 1.5 and 2.0%; based on recommended dose). Petri dishes containing amended medium were inoculated with 5mm disk of freshly prepared culture of *V. dahliae* Kleb under aseptic conditions. The un-amended PDA plates were treated as control. All the inoculated plates were incubated at 25±1°C. The data on mycelial colony growth was recorded at 10 days interval and final growth was recorded after 30days, when any one Petri dish established full fungal growth. The inhibition percentage of mycelial growth over control was calculated by using the formula suggested by Vincent (1947): I = (C-T)/C*100, Where, I = Per cent inhibition, C = Radial colony growth in control, T = Radial colony growth in treatment.

Greenhouse experiments

Series of experiments were performed two times under greenhouse conditions to find the effectiveness of treatments and the efficiency of different application techniques to reduce the *Verticillium* wilt development. Carbendazim (+), Topsin-M (+), Hymexazol (+) and PCNB (+) combinations and fungicides alone were applied with two different concentrations (low and high) in all application methods (Table 2). The treated and untreated seeds were sown in a 15 × 12-cm plastic pot with 10 seeds of each cotton germplasm being placed in each pot. The pots were filled with 1 kg of autoclaved sandy soil (sand and soil, 1:3) in each pot. The experiments were set out in a randomised complete block design (RCBD) with four replications and 4 factors being evaluated. These factors included germplasm, chemical compound and fungicides, concentrations and the application techniques. Plants were inoculated using soil drench methods to evaluate the efficacy of the all treatments. For soil drenching, 100 ml of spore/microconidia suspension (10^7 conidia ml^{-1}) was irrigated in each pot before sowing the seeds. Inoculated un-treated soil was considered as the control-1 and un-inoculated un-treated soil was considered as control-2.

Seed treatment and fungicides application

Seed of susceptible and tolerant germplasm were treated with fungicides combinations (+) and fungicides alone mixture. De-linted seeds were dipped in the solutions for an hour and air dried at room temperature for seed treatment (ST) method prior to sowing. Plants were treated two times; first application after 20 days of inoculation (DAI) and second at the interval of 15 days. In the greenhouse, four different methods of treatments were applied: 1) Without seed treatment (WST)+drench, 2) WST+spray, 3) ST+drench+spray, 4) ST+spray.

Assessment of treatments efficacy

The efficacy of treatments was assessed by using different growth parameters, wilt incidence (WI) percentage and infection percentage. Disease symptoms of *Verticillium* wilt

Table 1. List of the modern conventional fungicides and chemical compound

Name of fungicide	Chemical composition	Source
Bravo	75% Chlorothalonil WP	Syngenta Plant Protection Company, Su Zhou, P. R. China
Carbendazim	Carbendazim 50%WP	Fuda Agrochemical Co., Ltd., Jiangsu, P.R. China
Wilting powder (Huang ku jinfen)	Cu+Fe+Mn+Zn+B+Mo≥10.0% N+K2O≥14.5%	Ding Li Biological Co., Ltd., P.R. China
Hymexazol	Hymexazol 15%	Ji Qi Biochemistry Company, Gui Lin, P. R. China
Iprodione	Iprodione	Bayer Crop Science Co., Ltd., P. R. China
Pentachloronitrobenzene (PCNB)	Pentachloronitrobenzene WP40%	San Li Chemical Industry Co., Ltd., Shan Xi, P. R. China
Quadris	Azoxystrobin	Syngenta Plant Protection Company, Su Zhou, P. R. China
Thiram	Thiram 50% WP	Nantong the Baoye Chemical Co., Ltd.
Topsin-M	Thiophanate-methyl 70% WP	Jiangsu Longdeng Chemical Co., Ltd., Jiangsu, P. R. China
Bayleton or Triadimefon	Triadimefon	San Li Chemical Industry Co., Ltd., Shan Xi, P. R. China
SA	Sodium alginate	Aladdin Chemistry Co. Ltd., Shanghai, P.R. China
BHT	Butylated hydroxytoluene	Aladdin Chemistry Co. Ltd., Shanghai, P.R. China

Table 2. Concentrations of four conventional fungicides and chemical compounds used in greenhouse experiments

Fungicides	Recommended concentrations	Concentrations used	
		Low	High
Carbendazim	100-120g per 15L(Spray)	8g $1L^{-1}$ water (Spray)	8.5g $1L^{-1}$ water (Spray)
		5g$1Kg^{-1}$ seed (Seed treatment)	5.5g$1Kg^{-1}$ seed (Seed treatment)
		2g$1Kg^{-1}$ soil (Soil treatment)	2.5g$1Kg^{-1}$ soil (Soil treatment)
Hymexzol	20ml per 1000-1500X20 (Spray)	6.60ml $1L^{-1}$ water (Spray)	700ml $1L^{-1}$ water (Spray)
		5ml $1Kg^{-1}$ seed (Seed treatment)	5.5ml $1Kg^{-1}$ seed (Seed treatment)
		2ml $1Kg^{-1}$ soil (Soil treatment)	2.5ml $1Kg^{-1}$ soil (Soil treatment)
Topsin-M	100-200g per 100L water (Spray)	2g $1L^{-1}$ water (Spray)	2.5g $1L^{-1}$ water (Spray)
		5g$1Kg^{-1}$ seed (Seed treatment)	5.5g$1Kg^{-1}$ seed (Seed treatment)
		2g$1Kg^{-1}$ soil (Soil treatment)	2.5g$1Kg^{-1}$ soil (Soil treatment)
PCNB	100-200g per 100L water (Spray)	2g $1L^{-1}$ water (Spray)	2.5g $1L^{-1}$ water (Spray)
		5g$1Kg^{-1}$ seed (Seed treatment)	5.5g$1Kg^{-1}$ seed (Seed treatment)
		2g$1Kg^{-1}$ soil (Soil treatment)	2.5g$1Kg^{-1}$ soil (Soil treatment)
BHT		2g $1L^{-1}$	2.5g $1L^{-1}$
SA		2g $1L^{-1}$	2.5g $1L^{-1}$

Table 3. Analysis of variance for *in vitro test* and greenhouse experiments (wilt incidence, infection (%) and plant growth parameters treated with different fungicides alone and in combinations with SA and BHT) for the suppression of *V. dahliae* Kleb

Source	*In vitro* test		Wilt Incidence (WI) (%)		Infection %			Plant height (cm)	Fresh Plant weight (g)	No. of leave/plant
	DF	MS	DF	MS	DF	MS	DF	MS	MS	MS
Treatment	21	27657.5**	9	103182**	9	100870**	9	2825.98**	190.089**	1172.15**
Trial	1	55.2NS	1	14 NS	1	39 NS	1	2.88 NS	0.088 NS	0.93 NS
Dose	2	381.9**	1	10032**	-	-	1	317.52**	16.510**	99.14**
Method	-	-	3	1055	3	217	3	955.84	18.080	379.11
Variety	-	-	1	9262	1	7331	1	714.12	47.153	342.60
Replication	5	65.5	3	334	7	630	9	12.73	0.759	4.38
Treatment*Variety	-	-	9	1615	9	1264	27	30.37	0.643	13.78
Treatment*Method	-	-	27	169	27	108	9	7.35	0.630	1.84
Treatment*Dose	42	30.2	9	756	-	-	9	8.77	1.379	2.77
Error	720	20.8	1216	66	1192	71	3100	1.66	0.161	0.67
Total	791	-	1279	-	1279		3199	-	-	-
CV% (P<0.05)	-	5.93	-	31.10	-	24.55		5.12	23.69	19.29

** = Highly significant at P < 0.05 level
NS = Non significant
DF = Degree of freedom
MS = Mean Square
CV = Coefficient of variance

that were recorded in both germplasm included marginal chlorosis and/or necrosis of leaves, bronze colouration, one-sided leaf curl and wilting, as reported by Paplomatas *et al.,* (1992) after 15 days of each treatment. Individual plants that showed wilt symptoms were considered as diseased on the basis of a visual evaluation. Data were recorded for healthy and wilted plants, with the wilt incidence (%) of the disease being calculated using the following formula: Wilt Incidence (WI) = (Number of wilted plants/Total number of observed plants) * 100. The efficacy of all treatments was calculated by using the formula suggested by Abbott's formula (Abbot, 1925): Efficacy (%) = (X-Y)/X*100, Where, X= disease severity of the control, Y= disease severity of the treatment. Plant growth parameters, including plant height (cm), plant weight (g), and the number of leaves, were also recorded. Ten plants per replication were assessed for all plant growth parameters. Increase in biomass was calculated by using formula: Biomass increase (%) = [(fresh weights of plants treated-fresh weights of control plants)/fresh weights of control plants] *100. Re-isolation of the fungus was completed using inoculated-treated and inoculated-untreated plants to confirm the effectiveness of BHT and SA combinations and fungicide alone on the roots and stems of all treatments that were grown in pots. The percentage of infection was calculated using the following formula: Infection (%) = (Number of colonized tissues/Total number of inoculated tissues) * 100.

Table 4. Effect of different fungicides alone and in combinations with SA and BHT on the wilt incidence, infection (%) and plant growth inoculated with *V. dahliae* Kleb. under greenhouse conditions

Treatment	Dose	Wilt Incidence (WI)		Infection (%)*** of *V. dahliae*	Plant growth					
		(%)*	Treatment Efficacy (%)**		Height (cm)	Increase (%)****	Fresh Weight (g)	Increase (%)****	No. of leave/ Plant	Increase (%)****
Carbendazim	1	8.62 h	90.65 c	23.18 hi	27.07 e	25.97 e	2.11 f	75.76 de	5.52 d	12.81 d
	2	4.30 i	94.98 b	20.84 i	27.79 d	28.03 d	2.36 e	80.06 c	5.92 c	13.81 c
Carbendazim (+)	1	0.38 jk	99.57 a	0.00 j	29.86 a	33.11 a	2.98 b	84.64 a	7.17 a	16.93 a
	2	0.00 k	100.0 a	0.00 j	30.14 a	33.67 a	3.08 a	84.79 a	7.31 a	17.28 a
Hymexzol	1	32.22 e	52.79 gh	57.95 d	23.19 l	13.75 l	1.13 m	55.10 hi	2.96 l	6.410 k
	2	45.29 c	61.07 f	49.75 e	23.69 k	15.37 k	1.17lm	54.71 i	3.26 k	7.156 j
Hymexzol (+)	1	32.33 e	65.53 e	51.70 e	25.07 h	20.00 h	1.39 ij	61.50 g	3.97 i	8.938 h
	2	22.52 f	73.50 d	44.67 f	25.56 g	21.64 g	1.52 h	66.08 f	4.28 h	9.718 g
PCNB	1	42.77 c	44.34 i	63.03 b	22.16 n	10.42 n	1.18 m	57.16 h	2.22 n	4.646 m
	2	51.68 b	50.70 h	61.46 bc	22.66 m	11.70 m	1.24 kl	59.74 g	2.45 m	5.167 l
PCNB(+)	1	38.00 d	51.19 h	64.21 b	24.04 j	16.61 j	1.40 ij	65.36 f	3.21 k	7.052 j
	2	45.61 c	55.88 g	59.24 cd	24.53 i	18.29 i	1.42 i	64.06 f	3.51 j	7.781 i
Topsin-M	1	14.69 g	88.39 c	27.87 g	26.38 f	24.03 f	1.92 g	73.63 e	4.90 f	11.25 f
	2	4.663 i	90.10 c	24.75 h	26.87 e	25.55 e	2.08 f	76.65 d	5.17 e	11.93 e
Topsin-M (+)	1	3.155 ij	97.70 ab	0.00 j	28.31 c	29.25 c	2.59 d	81.34 bc	5.81 c	13.53 c
	2	1.38 jk	97.23 ab	0.00 j	28.80 b	30.56 b	2.77 c	83.17 ab	6.18 b	14.46 b
Contro-1		88.12 a	0.00 j	79.95 a	19.96 o	0.00 o	0.47 o	0.00 j	0.50 o	0.00 n
Control-2		0.00 k	-	0.00 j	23.79 jk		1.33 jk		5.15 e	

* = Wilt Incidence (WI) = (Number of wilted plants/Total number of observed plants) * 100.
** = Efficacy (%) = (X-Y)/X*100, Where, X= disease severity of the control, Y= disease severity of the treatment.
*** = Infection (%) = (Number of colonized tissues/Total number of inoculated tissues) * 100.
**** = Biomass increase (%) = [(fresh weights of plants treated-fresh weights of control plants)/fresh weights of control plants] *100.
1 = Low concentration
2 = High concentration
Control-1 = Inoculated un-treated
Control-2 = Un-inoculated Un-treated
"+" = means combination with BHT and SA

Note: Values sharing common letters regarding WI, treatment efficacy, infection (%), plant length, weight, number of leaves and increase (%) over control do not differ significantly at P < 0.05 according to least significant difference (LSD).

Table 5. Response of different treatment methods for the suppression of *Verticillium dahliae* under greenhouse condition

Methods	Wilt Incidence (WI)		Infection (%) of *V. dahliae*	Plant growth					
	(%)	Treatment Efficacy (%)		Height (cm)	Increase (%)	Fresh weight (g)	Increase (%)	No. of leave/Plant	Increase (%)
ST+Drench+spray	28.08 a	69.38 a	33.45 b	26.27 a	23.59 a	1.88 a	66.59 a	4.97 a	11.36 a
ST+Spray	27.40 a	69.32 a	33.96 ab	25.94 b	22.59 b	1.74 b	63.89 b	4.75 b	10.77 b
WST+ Drench+spray	24.66 b	65.91 b	35.00 a	24.30 c	16.90 c	1.59 c	59.63 c	3.71 c	7.88 c
WST +Spray	24.60 b	65.08 b	35.16 a	24.16 c	16.47 d	1.56 c	59. 62 c	3.64 c	7.68 c

ST = Seed treatment
WST = Without seed treatment

Note: Values sharing common letters regarding WI, treatment efficacy, infection (%) plant growth and increase percentage over control do not differ significantly at P < 0.05 according to least significant difference (LSD).

Statistical analysis

The *in vitro* and greenhouse experiments were repeated twice and combine analysis of both trials are presented. Data were statistically analysed according to standard procedures for analysis of variance, ANOVA (linear model), and mean separation (least significant difference, LSD) of all parameters including wilt incidence (WI), infection, efficacy, increase (%) over control (after calculating with corresponding formulas) and interaction between the trials were calculated by using the computer software Statistix 8.1 (Analytical Software, 2005). All differences described in the text were significant at the 5% level of probability.

RESULTS

In vitro efficacy of BHT and SA combinations against the V. dahliae *Kleb*

The efficacy of all tested fungicides and combinations with chemical compound (BHT and SA) exhibited varied response to reduce *V. dahliae* mycelial colony growth under laboratory conditions. All the tested treatments showed inhibitory effect, however, Carbendazim (+), Topsin-M (+), Hymexazol (+) and PCNB (+) combinations and fungicide alone showed significantly higher efficacy, to reduce 100% *V. dahliae* mycelial colony growth. The BHT and SA combination with Carbendazim (+), Topsin-M (+) also decolorized the mycelial plug due to their strongest efficacy compared to other treatments (Fig. 1). The effect of Huang ku jinfen(+) at higher and medium dose was also remained better compared to Bravo(+), Iprodione (+), Quadris (+), Thiram (+) and Triadimefon (+) combinations and alone treatments (Fig. 1). Analysis of variance showed the significant difference for treatments and doses at P < 0.05 level according to least significant difference (LSD), however, no significant difference was observed between two trials. Similarly, no significant difference was noticed for four fungicides (Carbendazim, Topsin-M, Hymexazol and PCNB) alone and their combinations with BHT and SA (Table 3)

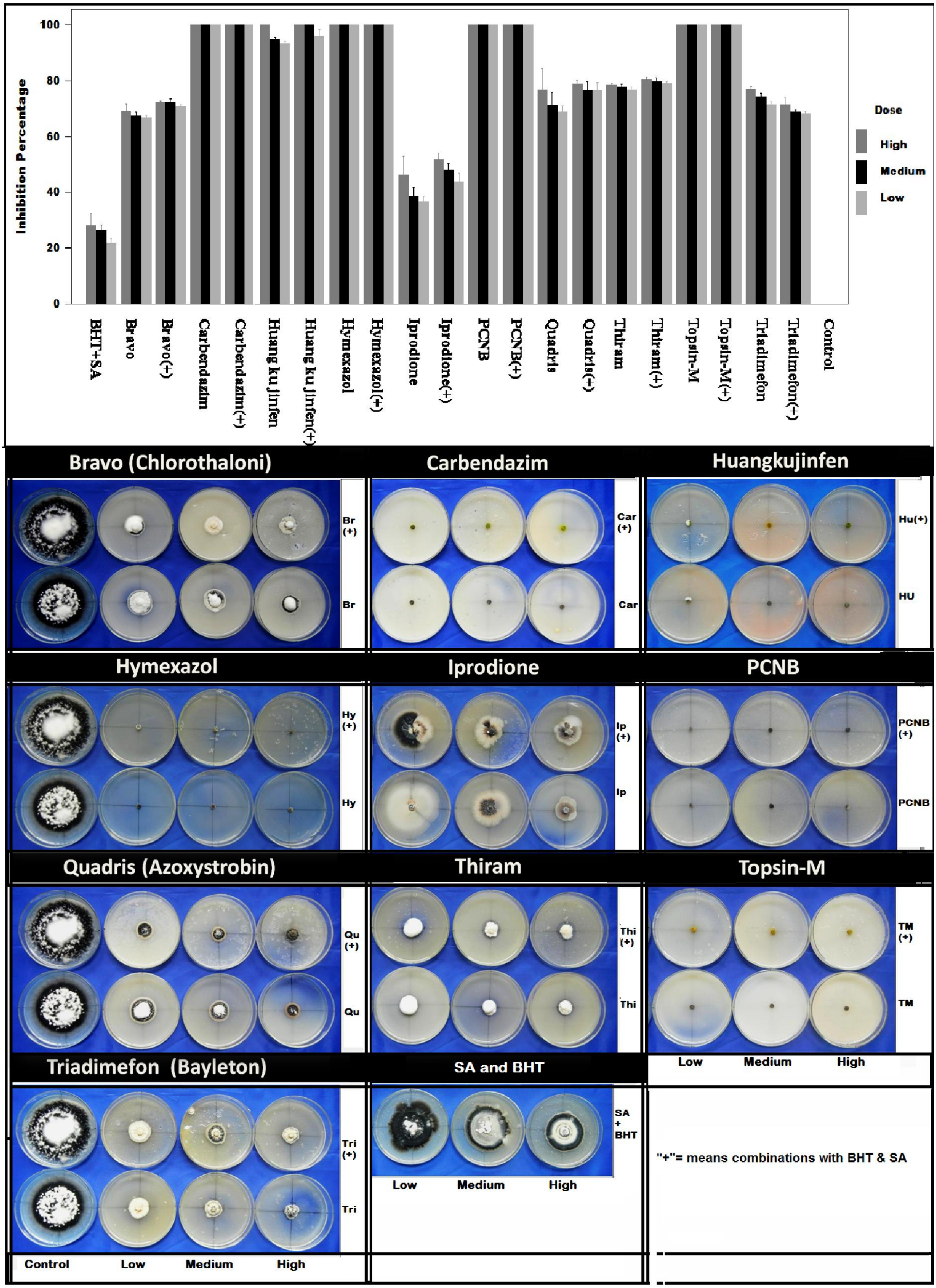


Figure 1. *In vitro* efficacy of SA and BHT along with ten different fungicides for the suppression of *V. dahliae* Kleb

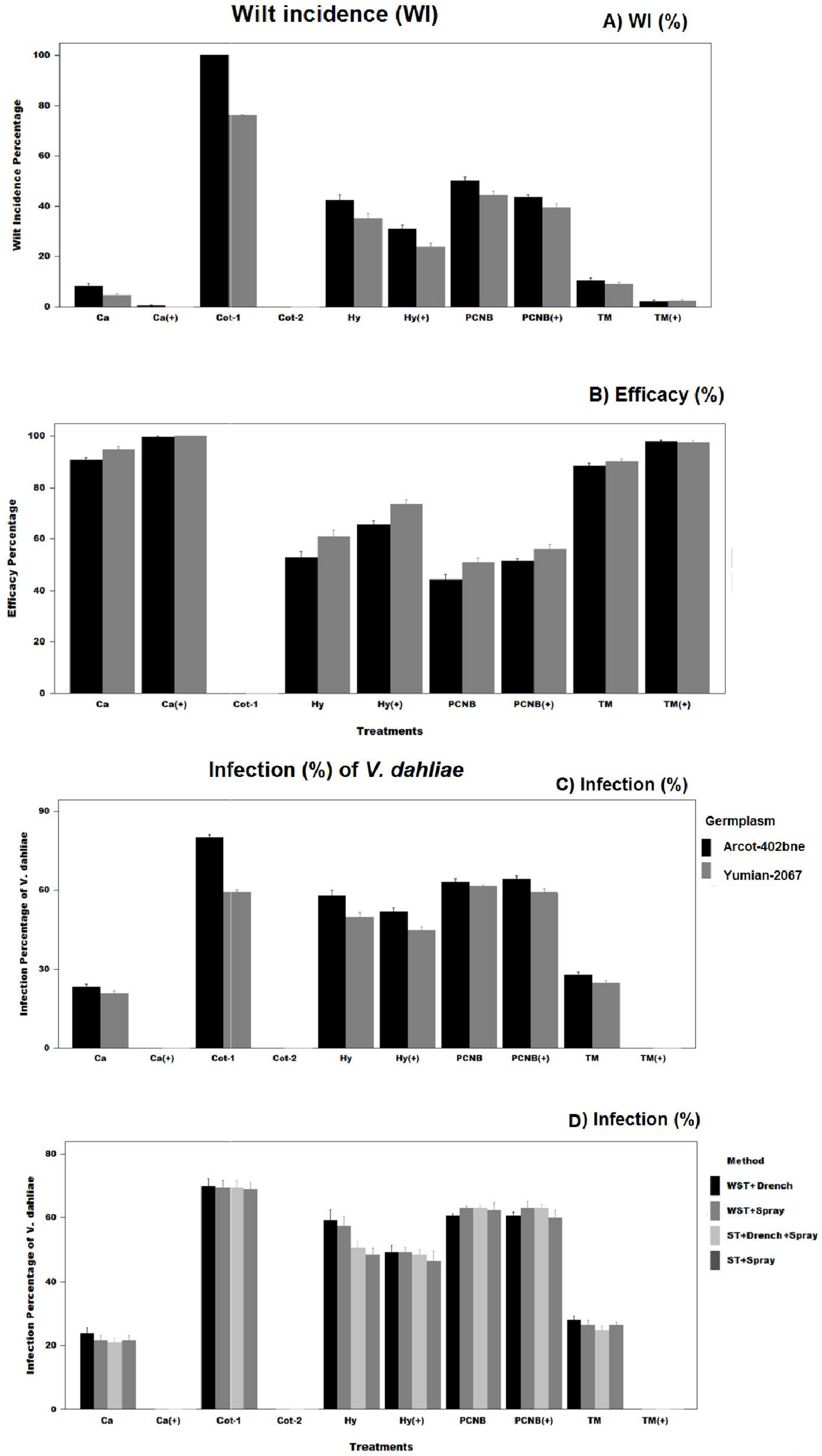
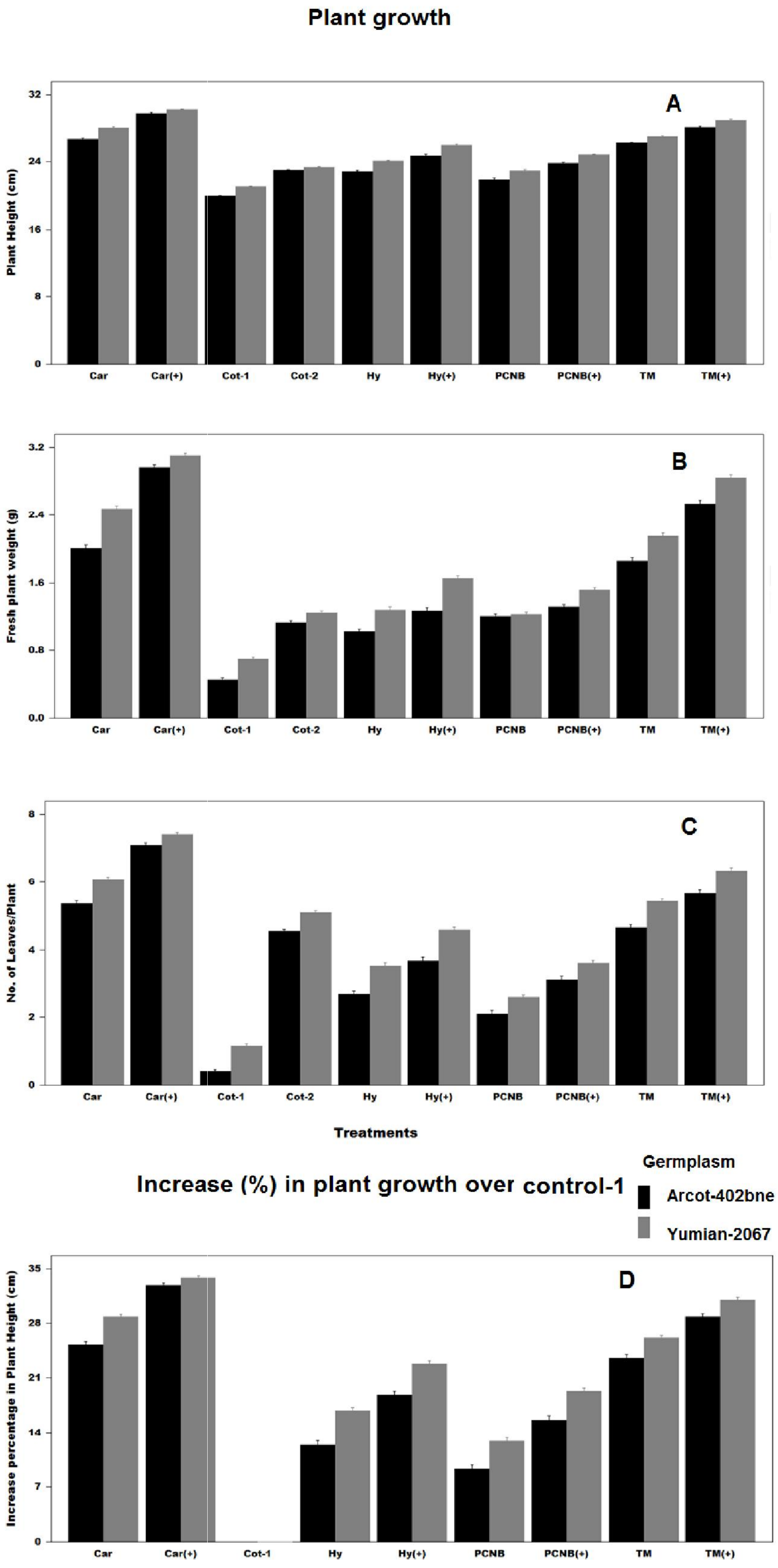


Figure 2. Wilt incidence and infection (%) of two cotton germplasm affected by SA and BHT combinations and fungicides alone under greenhouse conditions

__Note.__ A= WI (%) for two cotton germplasm; B= Efficacy of treatment for two cotton germplasm; C= Infection (%)for two cotton germplasm; D= Infection (%) of different treatment methods.

Ca = Carbendazim, Cot = Control, TM= Topsin-M, (+) = Combinations with BHT and SA


Plant growth
A
Plant Height (cm)
0
8
16
24
32
Car
Car(+)
Cot-1
Cot-2
Hy
Hy(+)
PCNB
PCNB(+)
TM
TM(+)
B
Fresh plant weight (g)
0.0
0.8
1.6
2.4
3.2
C
No. of Leaves/Plant
0
2
4
6
8
Treatments
Increase (%) in plant growth over control-1
Germplasm
Arcot-402bne
Yumian-2067
D
Increase percentage in Plant Height (cm)
0
7
14
21
28
35

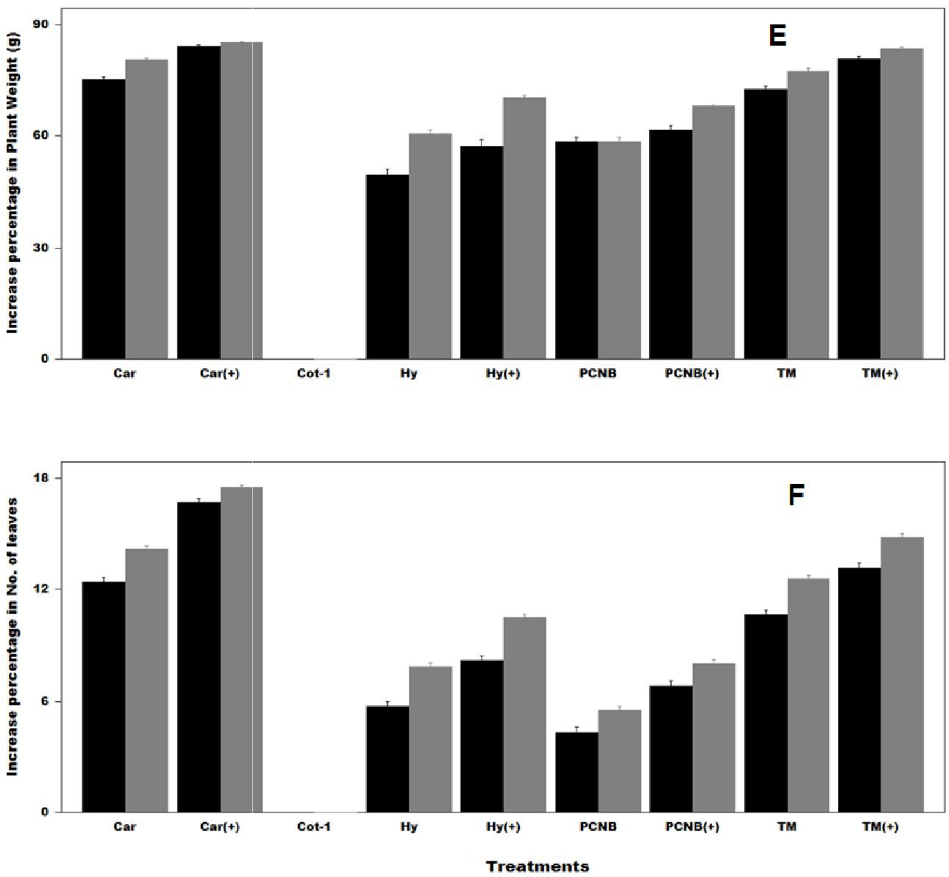


Figure 3. Plant growth of two cotton germplasm affected by SA and BHT combinations and fungicides alone under greenhouse conditions

Note. A= Plant height (cm); B=Plant weight (g); C= No. of leaves/plant; D, E,F= Increase (%) over control in plant height, weight and No. of leaves/plant, respectively.
Ca = Carbendazim, Cot = Control, TM= Topsin-M, (+) = Combinations with BHT and SA

Efficacy of BHT and SA combinations with Carbendazim and Topsin-M against V. dahliae *Kleb. under greenhouse*

Wilt incidence (WI). Analysis of variance for WI incidence percentage of tested cotton germplasm under greenhouse conditions varied significantly at $P < 0.05$ level among 8 different treatments (Table 3). Carbendazim (+) and Topsin-M (+) had the lowest WI percentage and showed strongest efficacy against *V. dahliae* Kleb. In contrast, Hymexazol (+), PCNB (+), Hymexazol and PCNB showed lower efficacy, with the highest WI percentage (Table 4). The germplasm Arcot-402bne and Yumian-2067 indicated different response for WI percentage with all treatments and is presented in Fig. 2. However, significantly lower WI percentage of two cotton germplasm was recorded with Carbendazim (+) and Topsin-M (+) (Fig. 2).

Infection percentage. *V. dahlia* Kleb was re-isolated from the root and stem pieces of all tested treatments under greenhouse conditions, to reconfirm the effectiveness of treatments for the suppression of *V. dahliae* Kleb growth in plant vascular systems (Table 4). *V. dahliae* Kleb extensively invaded inoculated-untreated (Control-1), PCNB, PCNB (+), Hymexazol and Hymexazol (+) treatments of susceptible and tolerant germplasm and produced a significantly higher infection percentage, indicates the low efficacy against *V. dahliae* Kleb. In comparison, the tissues of germplasm treated with Carbendazim (+) and Topsin-M (+) were not invaded by *V. dahliae* Kleb, proved their strongest efficacy against *V. dahliae* Kleb (Table 4, Fig. 2).

Plant growth. Results presented in Table 4 and fig. 3 demonstrates the effect of 8 different treatments on induction of plant growth resistance against *Verticillium* wilt development. Significantly higher plant height and fresh weight was recorded for Carbendazim (+) followed by Carbendazim and Topsin-M (+), whereas lower plant height and fresh weight was recorded for control-1 (inoculated) followed by PCNB, PCNB (+), Hymexazol and Hymexazol (+) (Table 4, Fig. 4). The increase in plant height and fresh weight compared to control-1 indicated significant variation among all 8 treatments at different doses is presented in Table 5, however, fig. 3 representing increase in plant height and fresh weight for two germplasm. Furthermore, the highest level of leaf defoliation was observed for PCNB, PCNB (+), Hymexazol and Hymexazol (+), indicates their lower effectiveness against *V. dahliae* Kleb. The increase percentage of leaves for Carbendazim (+) followed by Topsin-M (+), Carbendazim and Topsin-M was significantly higher compared to control-1, PCNB, PCNB (+), Hymexazol and Hymexazol (+) at two different doses (Table 4). These treatments showed high efficacy against *V. dahliae* Kleb for both germplasm (Fig. 4). However, Carbendazim (+) remained best due to its strongest effectiveness against *V. dahliae* Kleb. in all observed parameters (Table 4, Fig. 4).

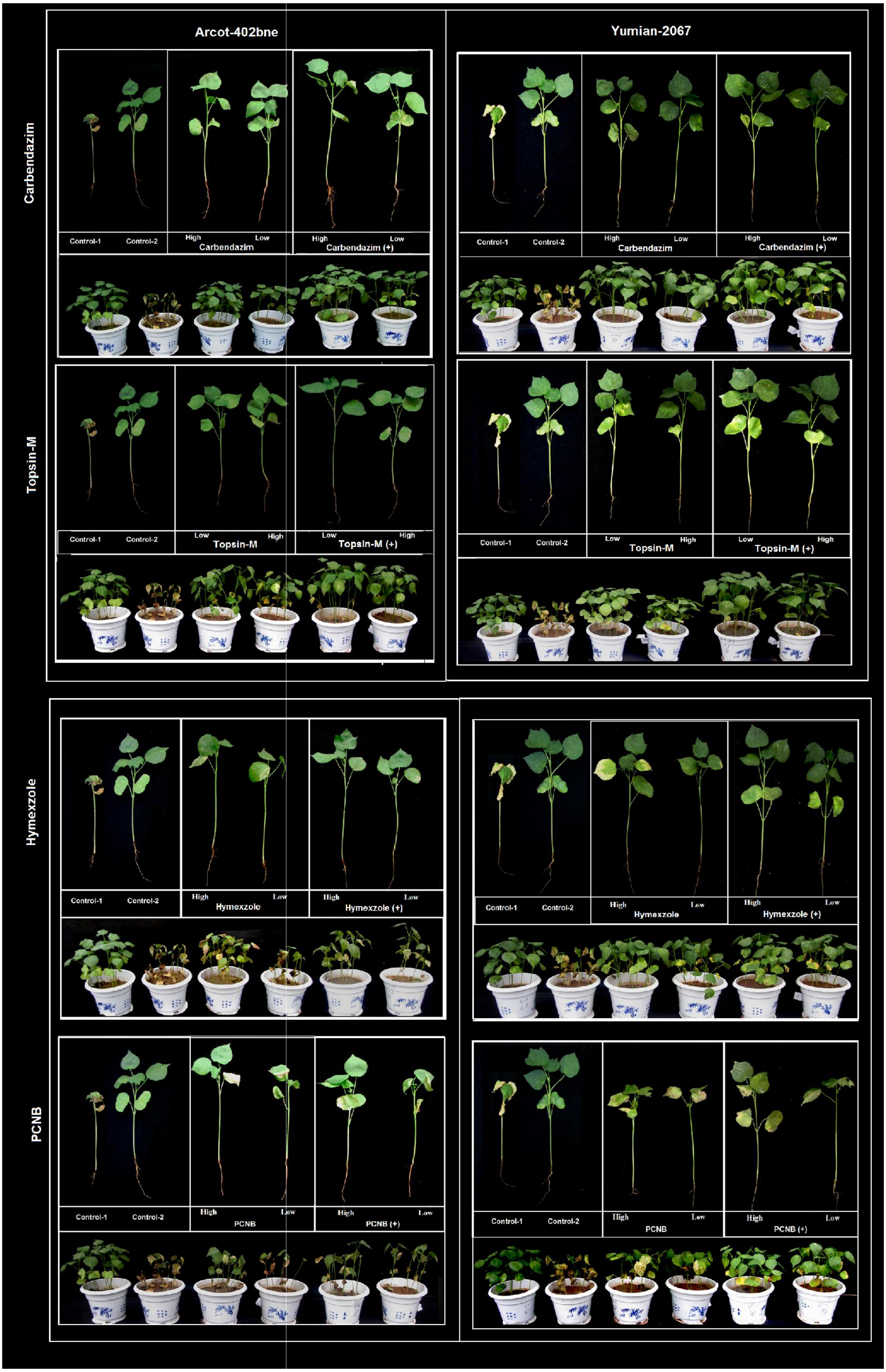


Figure 4. Response of SA and BHT combinations and fungicides alone under greenhouse conditions for plant growth

Effect of different treatment methods of application

It is evident from the results presented in Table 5 that ST+drench+spray and ST+spray resulted in the lower WI percentage and produced best performance compared to other methods. Significantly higher plant height, weight and number of leaves per plant were recorded with ST+drench+spray followed by ST+spray. Compared to the WST+drench and WST+spray techniques, significantly high efficacy of treatments were recorded by ST+drench+spray and ST+spray for the suppression of *Verticillium* wilt symptoms. ST+drench+spray and ST+spray were remained more effective compared to other methods; however, the latter method was more economically feasible and less time consuming (Table 5).

DISCUSSION

Why butylated hydroxytoluene (BHT) and sodium alginate (SA) combinations with the fungicides effectively suppressed the intensity of *V. dahliae* Kleb.? Here we discuss the potential use of BHT and SA combinations with conventional fungicides. The use of BHT and sodium alginate SA are well known with variety of purpose and application. However, to date these compounds have not been incorporated with other chemicals and fungicides for the control of *Verticillium* wilt. BHT is one of the synthetic antioxidant agents commonly used for food additives (Babu and Wu, 2008). However, it is also used; to increase the efficacy of organic biocides (Schultz *et al.,* 2006), as insecticide (Nesci *et al.,* 2011), as antifungal (Hsu *et al.,* 2007), anti-moulds (Elad, 1992) and as antioxidant (Babu and Wu, 2008; Ozen, 2011). The other chemical compound, SA is a natural polysaccharide, derived from brown algae and is available in large quantity. In the food industry, SA has the functions of stabilisation, hydration, thickening and emulsification. However, in agriculture, it can be used as seed treatment, insecticides and anti-viral materials (Tianjin Tiger International Trade Co., Ltd., Tianjin, China). SA has also been successfully used to stimulate plant growth, germination and shoot elongation in their depolymerised form for various plants (Aftab *et al.,* 2011; Idrees *et al.,* 2011; Khan *et al.,* 2011; Sarfaraz *et al.,* 2010).

Based on these facts, we incorporated BHT to enhance the antioxidant activities in cotton plant as well as to increase the efficacy of fungicides used against *Verticillium* wilt disease and SA as stabiliser to support the BHT. In addition, the use of conventional modern fungicides and other strategies are less effective. Thus effective management needs to be finding out to overcome this detrimental disease. In current study, we incorporated BHT and SA to enhance the efficacy of commercially available fungicides. Here, we discuss effective measures that may be used to induce high resistant with BHT and SA combinations in cotton germplasm through *in vitro* and greenhouse experiments. The *in vitro* test exhibited varied response to reduced *V. dahliae* mycelial colony growth due their effectiveness and compatibility with BHT and SA. Four combinations of Carbendazim, Topsin-M, Hymexazol and PCNB, which have been found more compatible with BHT and SA, effectively inhibit *V. dahliae* mycelial colony growth and proven as strongest and optimum chemical combinations under laboratory conditions. Interestingly, Carbendazim and Topsin-M combinations has typical and interesting response compared to other treatments by decolourising the mycelial plug of *V. dahliae* from blackish to yellowish colour. In the greenhouse experiments, when the highly susceptible (Arcot-402bne) and tolerant (Yumian-2067) germplasm were treated with 8 different treatments (4 strongest combinations viz: Carbendazim (+), Topsin-M (+), Hymexazol (+) and PCNB (+) incorporated with SA and BHT and 4 fungicides alone), inoculated with *V. dahlia*e Kleb, the effects on all observed parameters has significantly varied at $P < 0.05$ level. The response of treatments used in greenhouse experiments, are correlated with *in vitro* test especially for Carbendazim (+) and Topsin-M (+) combinations, and Carbendazim and Topsin-M alone. However, with the incorporation of SA and BHT, Carbendazim and Topsin-M has resulted the strongest efficacy in the reduction of *Verticillium* wilt symptom development and intensity of *V. dahlia*e Kleb. All the observed parameters were greatly influenced by the effect of these combinations. Maximum plant height and weight, lower WI and infection percentage has resulted by Carbendazim and Topsin-M combinations, indicates their high effectiveness. However, the effect of Hymexazol and PCNB combinations were also remained better compare to alone fungicide treatments.

In addition, Carbendazim and Topsin-M combinations were remained stable under greenhouse compare to Hymexazol and PCNB combinations due to their structural compositions. However, when Hymexazol and PCNB fungicides were used alone, had also poor response under greenhouse conditions that might be due to lower efficacy and stability of these fungicides; and/or survival and capability of *V. dahlia*e Kleb. It is also evident from the previous studies that use of conventional fungicides (Talboys, 1984; Tian *et al.,* 1998) are also less effective due to survival and capability of *V. dahlia*e Kleb. in the soil for long time as mycelia and microsclerotia; and quick dispersal through various means of agricultural practices (Rekanovic *et al.,* 2007). In contrast, the use of some modern fungicides and other products to reduce *V. dahliae* growth and disease development are also reported. In particular, a strobilurin fungicide, azoxystrobin (Quadris) (Crowe and Simmons, 2007) and benomyl, (Rekanovic *et al.,* 2007) are reported to prevent all visual wilt symptoms.

However, variability in the efficacy of benzimidazoles (Benomyl) is also reported depending on the duration and type of application, concentration, and individual host plant response (Beckman, 1987). Here, we also found the variability in the efficacy of combinations and alone treatments with different techniques and doses. The results of Quadris in our study are inconsistent with Rekanovic *et al.,* (2007) and here we found less effective against or to reduce *V. dahlia*e Kleb. growth and severity. The method, dosage and timing of applications play an important role in efficacy of fungicide treatments against diseases control. In our *in vitro* test the response of lower and higher doses of four fungicides alone (Carbendazim, Topsin-M, Hymexazol and PCNB) and their combinations has the similar inhibition response due to their higher effectiveness. Similarly, no significant difference between higher and lower doses was observed for Carbendazim and Topsin-M under greenhouse test. However, lower doses are more economically feasible, can be used for higher scale. In the present study, ST+drench+spray and ST+spray proved best management approach and lowered the WI percentage, produced significantly maximum plant height and weight, number of leave per plant. Although, ST+drench+spray and ST+spray are better methods, however, the latter method is more economically feasible, less time consuming and easiest than others. In a similar study, Carbendazim (benzimidazole) fungicide provides better control of *Verticillium* wilt when applied by drenching (Buchenauer *et al.,* 1971). The present study determined strongest and optimum chemical combination (Carbendazim+BHT+SA and Topsin-M+BHT+SA) with high efficacy and great potential, to suppress the *Verticillium* wilt development, which is the major threat to the yield and quality of cotton crop. It is hoped that these new combinations will effectively control this serious disease and improve the cotton quality for sustainable production and ultimately benefit the end user (grower and manager).

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