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Research Article

Frequency of transposable elements and fungicide resistance in *Botrytis Cinerea* Pers. populations on strawberries from Aydin and Mersin provinces in Turkey

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ABSTRACT

Strawberry is a delicious fruit that can be consumed fresh or/and can be used in the industry. One of the important factors that affect yield is the plant protection problems. Fungicides because of the metabolites they produce, especially their presence in foods, pose a threat to health and also cause economic problems. Botrytis cinerea causes grey mold disease in over 220 plant species. Samples were collected from five different localities from Aydın and Mersin province. Samples were incubated on um and then isolated from mixed cultures. Morphological and molecular identification of the samples were made. Transposon sites were amplified with PCR. In total 154 *B.cinerea* samples were isolated of which *Transposa* 20.1%, *Boty* 46.1%, *Flipper* 10.4% and *Vacuma* 23.4%, in Aydın population *Transposa* 19.5%, *Boty* 48.3%, *Flipper* 9.2% and *Vacuma* 23.0%, in Mersin *Transposa* 20.9%, *Boty* 43.3%, *Flipper* 11.9% and *Vacuma* 23.9% were found. In the fungicide resistant test, in spore tests, Cyprodinil was found most effective while fenhexamid was found less effective. In mycelium tests, fenhexamid was most effective at the lowest concentration applied while carbendazim was found less effective even at the highest concentration. Although short-term results are limited, in long-term transposon profiling can help with disease management.

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INTRODUCTION

Strawberry (*Fragaria vesca*) is a perennial plant that belongs to *Rosaceae* family. Strawberry fruits can be consumed raw or can be used in industry. One of the most important factors that effect the yield is plant protection problems. Products are exposed to many disease factors both pre and post-harvest. One of these factors is Grey mold disease caused by *B. cinerea* Pers. It affects more than 220 plant species resulting in huge health and economic

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problems [1]. It shows its effects especially in cool and moist conditions. It can affect all plant parts but most important damages are caused on fruit [2]. Control of grey mold is fungicide application in different times of year but *B.cinerea* can adapt to the fungicides quickly and develop resistance. This genome plasticity and evolution can be explained with transposons [3] and inteins [4]. Although *B.cinerea* has both sexual and asexual stage in its life cycle sexual stage can rarely seen in nature [5].

Agricultural warfare is the protection of plants from pests, diseases and other factors. Chemical warfare is most frequently used method, because of cost effectiveness [6]. Because of high adaptability of the fungus, accumulation of these fungicides in fruits due to its over use causes health problems to consumers. Many fungal agents cause disease in different parts of strawberries. Fungicides can be used for preventive purposes; they can partially suppress disease [7]. However, researchers showed quick resistance development against fungicides [8].

Transposons are DNA sequences which can change their locations on the genome. They have in different mechanisms and structures such as retrotransposons and DNA transposons [9–11] Both retrotransposons and DNA transposons can be found in *B.cinerea* [12]. They are recognized as a potential cause of structural rearrangements of DNA sequences and chromosomes and changes in gene expression [12]. Their presence in the genome of *B.cinerea* might explain genetic variability and is considered as an additional source of diversity. *B.cinerea* was considered to be a single generalist species until the identification of two transposable elements; *boty* [13] and *flipper* [14]. These discoveries led to a grouping based on presence or absence of these two transposons. Four groups were formed based on these two elements: *transposa* (*boty* and *flipper* transposons are present), *boty* (only *boty* transposon present) and *flipper* (only flipper transposon is present), *vacuma* (neither *boty* nor *flipper* are present) [15]. These groups can be detected easily with a PCR based method.

Several studies were made after discovery of these groups. Among these studies, Ma and Michailides [16] studied genetic structures of *B.cinerea* in California, Kretschmer and Hahn [17] studied genetic diversity and fungicide resistance of *B.cinerea* populations from vineyards in Germany, Samuel et al [18] studied the frequency of transposable elements in *B.cinerea* from several hosts in Greece, and Fekete et al [19] studied genetic diversity of *B.cinerea* in Hungary. Van Kan et al. [20] reported a gapless genome sequence of *B.cinerea* strain B05.10. Petrasch et al. [21] reviewed the biology, epidemiology, mechanisms of infection and the genetics of host plant resistance. Abbey et al [22] reviewed biofungicides that can be alternative to synthetic fungicides and focused on *Trichoderma* spp, *Ulocladium* spp, *Bacillus subtilis*, extracts of *Ephedra breana* and *Nolana sedifolia*.

The aim of this study is to determine distribution and frequency of transposon in genomes of *B.cinerea* isolates from Aydın and Mersin provinces and to find out fungicide resistances based on transposon groups.

MATERIALS AND METHODS

Sample Collection

Samples were collected aseptically from Aydın and Mersin provinces in December 2015 and April 2016. Three locations from Aydın (Atça, Sultanhisar and Yenipazar) and two locations from Mersin (Anamur and Silifke) were chosen due to their high amount of strawberry production. At least 20 fruits that show symptoms of grey mold were collected from each location.

Isolation and Identification of *Botrytis cinerea*

One gram of each diseased fruit was weighted and homogenized in 9 mL of physiological saline water (PSW). Then samples were inoculated on Potato Dextrose Agar (PDA) and incubated for 20°C 5 days. This process is repeated until *B.cinerea* colonies isolated from mixed cultures. Morphological identification of the isolates was made according to Samson et al [23].

Molecular identification of *B.cinerea* isolates was made with rDNA ITS regions. Genomic DNA isolation was made according to 2X CTAB DNA isolation method [24]. A set of ITS1(5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT GCT TTA TTG ATA TGC-3′) primers

Table 1. PCR protocols used in the study.

PCR Protocols used in the study					
	ITS	**Boty**	**Flipper**	**Bc-hch**	
In.Denaturation	95°C 3 m	95°C 3 m	95°C 3 m	95°C 3 m	
Denaturation	94°C 30 s	94°C 30 s	94°C 40 s	94°C 40 s	
Annealing	58°C 30 s	64°C 30 s	60°C 40 s	51°C 40 s	X 35
Extension	72°C 60 s	72°C 60 s	72°C 60 s	72°C 60 s	
Final Extension	72°C 10 m	72°C 3 m	72°C 10 m	72°C 10 m	

were used. PCR protocol can be seen in Table 1. PCR products were observed on 1.4% agarose gel at 90 V 40 min. Then they were sent to sequencing (Macrogen, Holland). ITS sequences were aligned with the ones in the GenBank using BLASTn software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Transposon Detection

Two transposon regions, *boty* and *flipper*, were amplified using PCR method. Two primer pairs were used: Boty F4 (5′- CAG CTG CAG TAT ACT GGG GGA-3′) and BotyR4 (5′- GGT GCT CAA AGT GTT ACG GGA G-3′) for *boty* transposon [13], and F300 (5′-GCA CAA AAC CTA CAG AAG A-3′) and F1550 (5′- ATT CGT TTC TTG GAC TGT A-3′) for *flipper* transposon [14,16]. The amplification protocol for *boty* and *flipper* can be seen in Table 1. PCR products of transposons were controlled on 1.4% agarose gel at 90 V 40 min.

***Bc-hch* Amplification and Digestion**

PCR-RFLP of the *Bc-hch* gene was used to identify group I and group II isolates according to Fournier et al. [25]. Two primers: 262 (5′- AAGCCCTTCGATGTCTTGGA-3′) and 520L (5′ - ACGGATTCCGAACTAAGTAA-3′) were used to amplify *Bc-hch* gene region. The amplification protocol for *Bc-hch* can be seen in Table 1. Digestion was carried out on 10 µL of PCR product, 2 µL of Hha I (Invitrogen). Incubation was performed for 2 h at 37°C. Results of RFLP was visualized on 1.4% agarose gel at 90 V 40 min.

Fungicide Resistance Tests

Three fungicides were chosen for resistance tests, fenhexamid, carbendazim and cyprodinil, due to their frequent use against *B.cinerea*. Fungicide resistance tests were made with 2 stages: spore stage and mycelium stage. Spore stage experiments were carried away according to Vercesi et al [26]. HA broth (10 g Malt extract, 4 g yeast extract, 4 g glucose per liter, pH 5.5) was prepared with following fungicide concentrations for each fungicide: 0, 0.1, 0.3, 1, 3 µg/ mL, determined in the previous studies [17,27,28]. From each isolate group, 2×10^5 spore/ml spore were pre-incubated in fungicide-free HA broth for 1.5 h at 20°C. Fifty microliters of suspensions were mixed with 950 µL fungicide containing HA broths. Assays were incubated at 20°C 48 h. Evaluation was performed with a spectrophotometer (Shimadzu) at 492 nm. Growth Inhibition Percentage (GIP) was calculated according to the following formula:

$$[(AC_{t2} - AC_{t0}) - (AF_{t2} - AF_{t0})] \times \frac{100}{(AC_{t2} - AC_{t0})} \quad (1)$$

Mycelium stage experiments were conducted according to Fekete et al [19]. From each fungicide different concentrations were prepared with PDA (Table 2). Mycelium from each group were taken and inoculated on fungicide containing PDAs. Samples were incubated at 20°C for 72 h. After incubation colony radius was measured and GIP values were calculated as accepting the control as 0%. All statistical analysis was made with IBM SPSS v22. After fungicide tests IC_{50} values were calculated using the calculation engine at http://www.ic50.tk/

Table 2. Fungicide concentrations prepared for mycelium stage fungucide resistance tests

Fungucide concentrations prepared for mycelium tests (mg/mL)		
Fenhexamid	**Carbendazim**	**Cyprodinil**
5000	1250	400
500	1000	200
50	750	100
25	75	50
12.5	50	25
10	7.5	20
5	5	10
2	2.5	0
0	0	–

RESULTS AND DISCUSSION

We confirmed that our isolates are *B.cinerea* by ITS sequencing. ITS sequences were aligned using BLASTn tool. Isolates identity percentage with *B.cinerea* 97% and higher were accepted. One hundered fifty four isolates meet the criteria and selected for further experiments.

Transposon PCR results showed that among 154 *B.cinerea* samples 31 of them were found *transposa*, 71 of them were *boty*, 16 of them were *flipper* and 36 of them were *vacuma* isolates (Table 3, Fig 1, 2).

Statistics based on the location were given in Table 4 and Fig 3. Location*group cross tabulation and chi-square tests were given in Table 5.

We isolated 154 *B.cinerea* isolates and identified 31 *transposa*, 71 *boty*, 16 *flipper* and 36 *vacuma* groups among these 154 isolates. The percentage of group frequencies are as follows: 20.1%, 46.1%, 10.4% and 23.4%, respectively. Kreschmer and Hahn [15] found out *transposa* as the first group with 30% while the second group was found as *boty* with 23.5%. *Vacuma* was the third with 14.4% while they didn't find any *flipper* group. Samuel et al. [16] found from most to least frequent, *transposa*, *vacuma, flipper* and *boty*. Fekete et al [17] in Hungary, found *boty, transposa, vacuma* and *flipper* from most to least frequent. Asadollahi et al. [29] in Hungary, found *transposa, flipper, boty* and *vacuma* from most to least frequent. Kumari et al. [30] in India, found *boty, flipper, vacuma* and *transposa*, respectively. Population

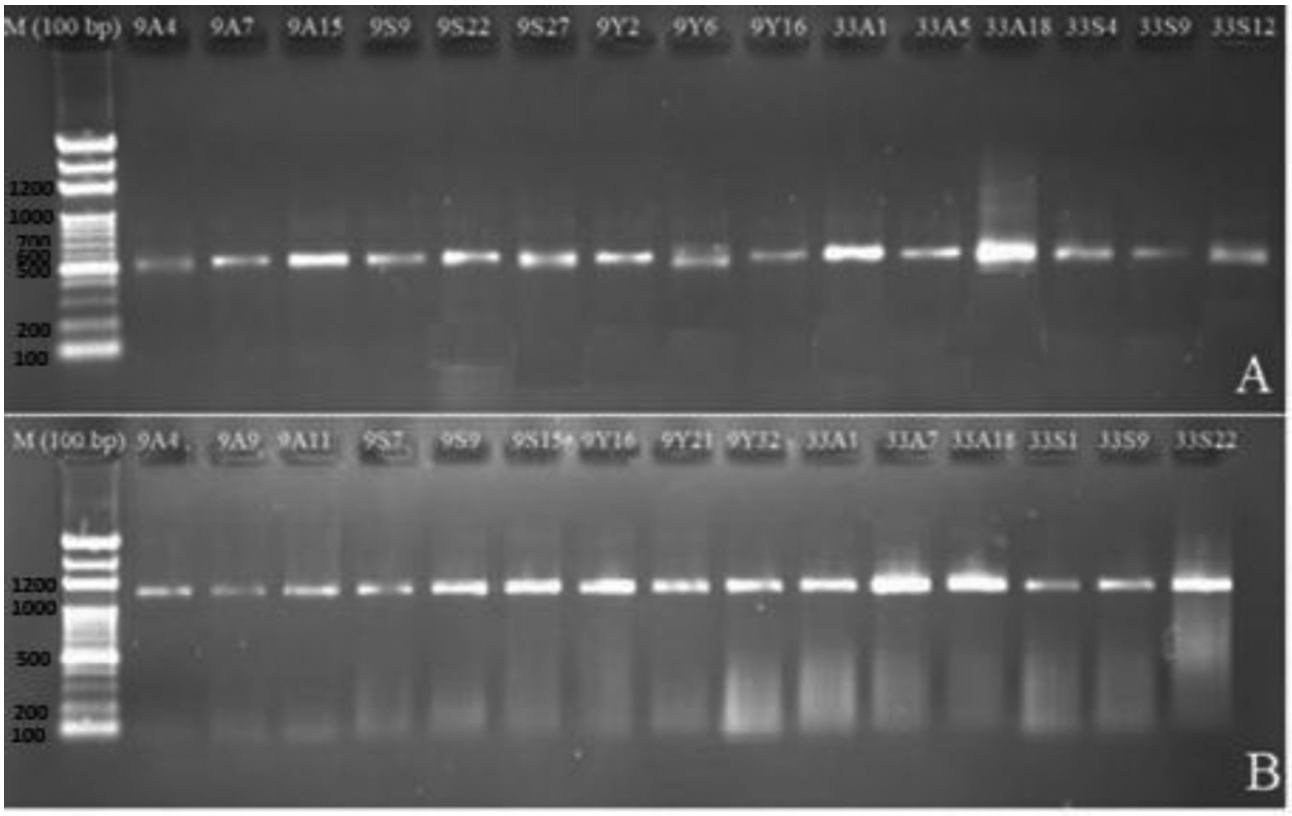

Figure 1. Transposon PCR results of selected samples. A: *boty*, B: *flipper*, 9AX: Atça, 9SX:Sultanhisar, 9YX: Yenipazar, 33AX: Anamur, 33SX: Silifke.

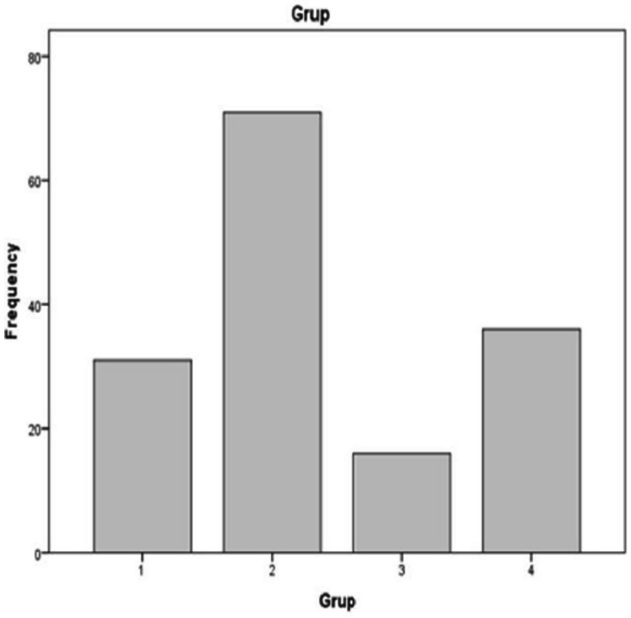

Figure 2. Frequency graphic of transposon groups based on number of strains for each group. 1:Transposa, 2: Boty, 3: Flipper, 4: Vacuma.

Table 3. Tranposon group frequencies and percenteges. 1:Transposa, 2: Boty, 3: Flipper, 4:Vacuma

Transposon group frequencies and percentages				
	Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
1	31	20,1	20,1	20,1
2	71	46,1	46,1	66,2
3	16	10,4	10,4	76,6
4	36	23,4	23,4	100,0
Total	154	100,0	100,0	

structure based on transposon groups can change from one country to another since transposon structure can even differ in the same location in different years. If we look at transposon groups based on locations, a close frequency distribution to the overall frequencies can be seen. All transposon groups were found in each location except Atça population where *flipper* group wasn't seen. Kumari et al. [30] had also seen a similar situation in their studies

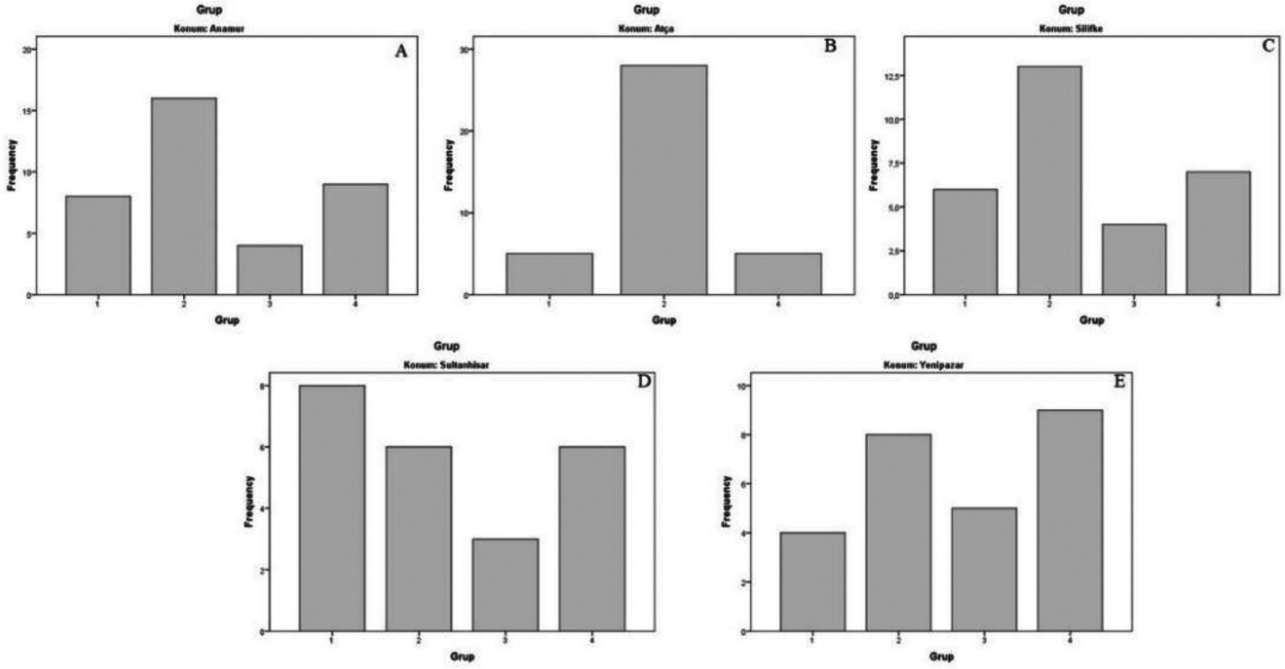

Figure 3. Transposon PCR results of selected samples. A: *boty*, B: *flipper*, 9AX: Atça, 9SX:Sultanhisar, 9YX: Yenipazar, 33AX: Anamur, 33SX: Silifke.

Table 4. Frequencies and percentages of groups based on sampling location. 1:Transposa, 2: Boty, 3:Flipper, 4:Vacuma

Location			Frequency	Percent	Valid Percent	Cumulative Percent
Anamur	Valid	1	8	21,6	21,6	21,6
		2	16	43,2	43,2	64,9
		3	4	10,8	10,8	75,7
		4	9	24,3	24,3	100,0
		Total	37	100,0	100,0	
Atça	Valid	1	5	13,2	13,2	13,2
		2	28	73,7	73,7	86,8
		4	5	13,2	13,2	100,0
		Total	38	100,0	100,0	
Silifke	Valid	1	6	20,0	20,0	20,0
		2	13	43,3	43,3	63,3
		3	4	13,3	13,3	76,7
		4	7	23,3	23,3	100,0
		Total	30	100,0	100,0	
Sultanhisar	Valid	1	8	34,8	34,8	34,8
		2	6	26,1	26,1	60,9
		3	3	13,0	13,0	73,9
		4	6	26,1	26,1	100,0
		Total	23	100,0	100,0	
Yenipazar	Valid	1	4	15,4	15,4	15,4
		2	8	30,8	30,8	46,2
		3	5	19,2	19,2	65,4
		4	9	34,6	34,6	100,0
		Total	26	100,0	100,0	

Table 5. Group- Location crosstabulation and chi-square tests. 1:Transposa, 2: Boty, 3:Flipper, 4:Vacuma

Group * Location Crosstabulation and Chi-Square							
		Location					
		Anamur	**Atça**	**Silifke**	**Sultanhisar**	**Yenipazar**	**Total**
Group	1	8	5	6	8	4	31
	2	32	56	26	12	16	142
	3	12	0	12	9	15	48
	4	36	20	28	24	36	144
Total		88	81	72	53	71	365
		Value		df		Asymp. Sig. (2-sided)	
Pearson Chi-Square		54,400[a]			12	,000	
Likelihood Ratio		62,447			12	,000	
N of Valid Cases		365					

a. 1 cells (5,0%) have expected count less than 5. The minimum expected count is 4,50.

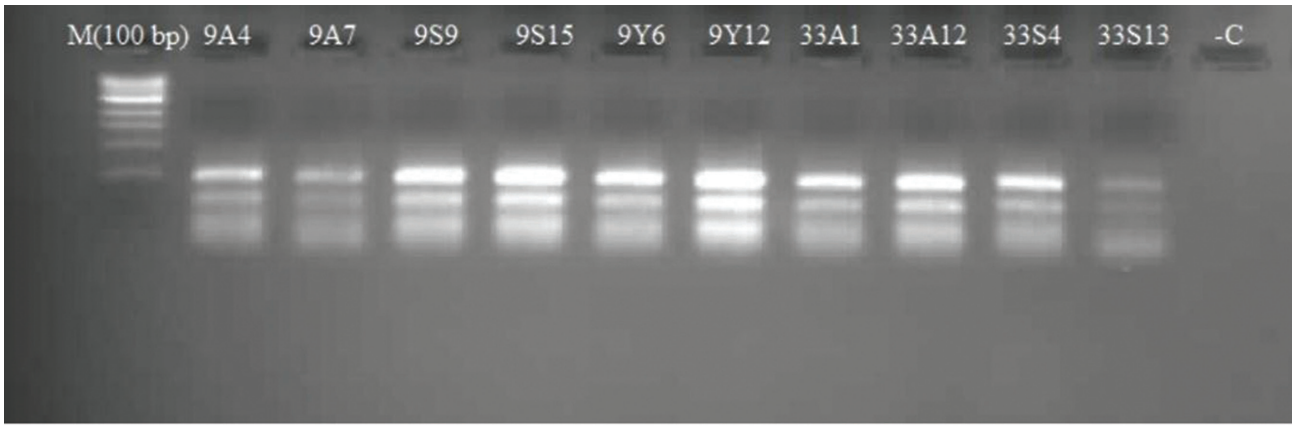

Figure 4. Hha I RFLP pattern of *Bc-hch* gene of selected samples. M: Marker, -C: Negative Control, 9AX: Atça, 9SX:Sultanhisar, 9YX: Yenipazar, 33AX: Anamur, 33SX: Silifke.

which they didn't find any *boty* and *transposa* in East India population. According to the chi-square test observed value was higher than the expected value. Also, p value was found lower than 0.05, it can be said there is a meaningful relation between group and location. If we evaluate the transposon structure based on provinces, *boty* was found 5% higher in Mersin than Aydın. For other groups difference is lower than 1.5%.

Group I isolate are less frequently in Europe [16,25] than Group II isolates as studies, including ours, suggests. Fekete et al. [19] suggested that group II isolates contain all transposon groups while group I isolates consists of *vacuma* group. Since all our isolates belongs to Group II, we have found all transposon groups but we can't make any interpretations about Group I isolates only consists of *vacuma* since all our samples belongs to group II.

Group I isolates contains 4 Hha I restriction sites while group II isolates contains 5 restriction sites. All our isolates belongs to group II (Fig 4).

There are a few studies about *Bc-hch* groups and transposable elements are related to fungicide resistance [31–33]. Suty et al [34] reported Group I isolates metabolizes fenhexamid faster. Although we didn't conduct a study related to this, from the literature, we can interpret transposons may have regulatory effects on genes or processes related to fungicide resistance.

After spore stage experiments GIP values were calculated (Table 6; Fig 5). Cyprodinil showed the highest effect (89%<) while fenhexamid found least effective (24% – 89%).

For spore stage resistance tests cyprodinil was found most effective (>87%); followed by carbendazim (39% – 98%) and lastly fenhexamid (24% - 89%). For transposon

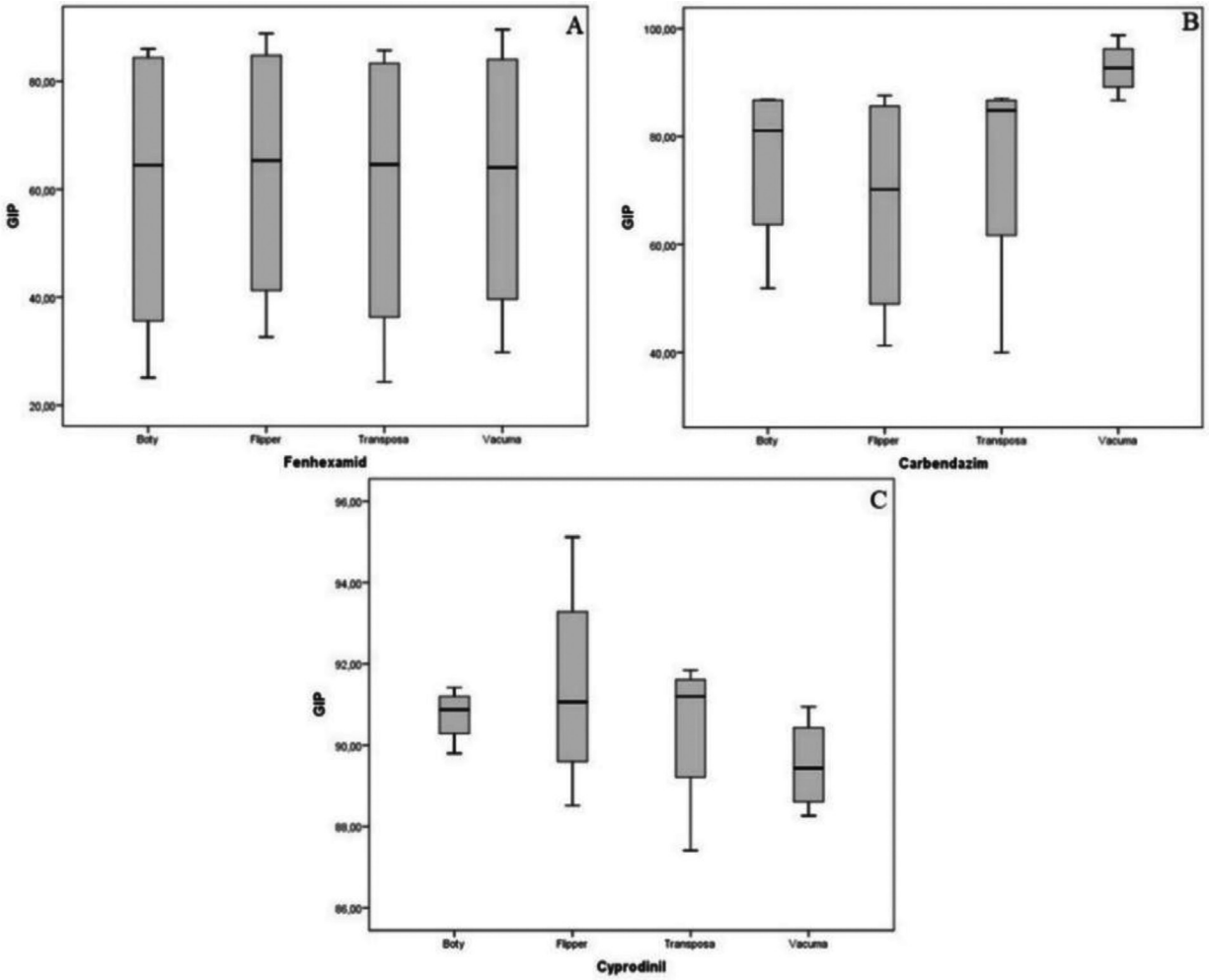

Figure 5. GIP value graphic of fungucides for spore stage tests. A: Fenhexamid, B: Carbendazim, C: Cyprodinil.

Table 6. GIP values of fungicides for spore stage tests

Cons.	Transposa	Boty	Flipper	Vacuma
Fenhexamid GIP values				
3 µg/mL	85.745	86.034	88.857	89.582
1 µg/mL	80.866	82.777	80.737	78.505
0.3 µg/mL	48.316	46.148	48.316	49.517
0.1 µg/mL	24.297	25.088	24.297	29.791
Carbendazim GIP values				
3 µg/mL	87.056	86.602	87.603	98.736
1 µg/mL	86.302	86.655	83.706	93.735
0.3 µg/mL	83.374	75.532	56.702	91.640
0.1 µg/mL	39.980	51.886	41.239	86.693
Cyprodinil GIP values				
3 µg/mL	91.844	91.420	91.544	90.945
1 µg/mL	91.377	90.978	91.377	89.916
0.3 µg/mL	91.018	90.770	91.018	88.950
0.1 µg/mL	87.513	89.796	87.407	88.264

groups, for Cyprodinil, resistance is found from high to low *transposa, boty, flipper* and *vacuma*, respectively. For carbendazim *vacuma, flipper, transposa* and *boty*, respectively. For fenhexamid *vacuma, flipper, boty* and *transposa* from high to low, respectively.

Mycelium stage experiments results were shown in Tables 7. Fenhexamid showed an effect at the lowest concentrations (5 mg/L) while carbendazim showed an effect at highest concentrations (1250 mg/L). Cyprodinil has shown its effect at 20 mg/L concentration. GIP graphics of mycelium stage was shown in Fig 6.

IC_{50} values were found at the lowest for fenhexamid while highest for carbendazim. IC_{50} values based on transposon groups were also shown in Table 8.

In mycelium stage effects of the all fungicides, from high to low, *flipper, transposa, vacuma* and *boty* were observed. Effective concentrations were varied according to the fungicide. Although fenhexamid was effective at the lowest concentrations, Delen [6] reported that field effects of fenhexamid weren't as effective as *in-vitro* effects. It is due to

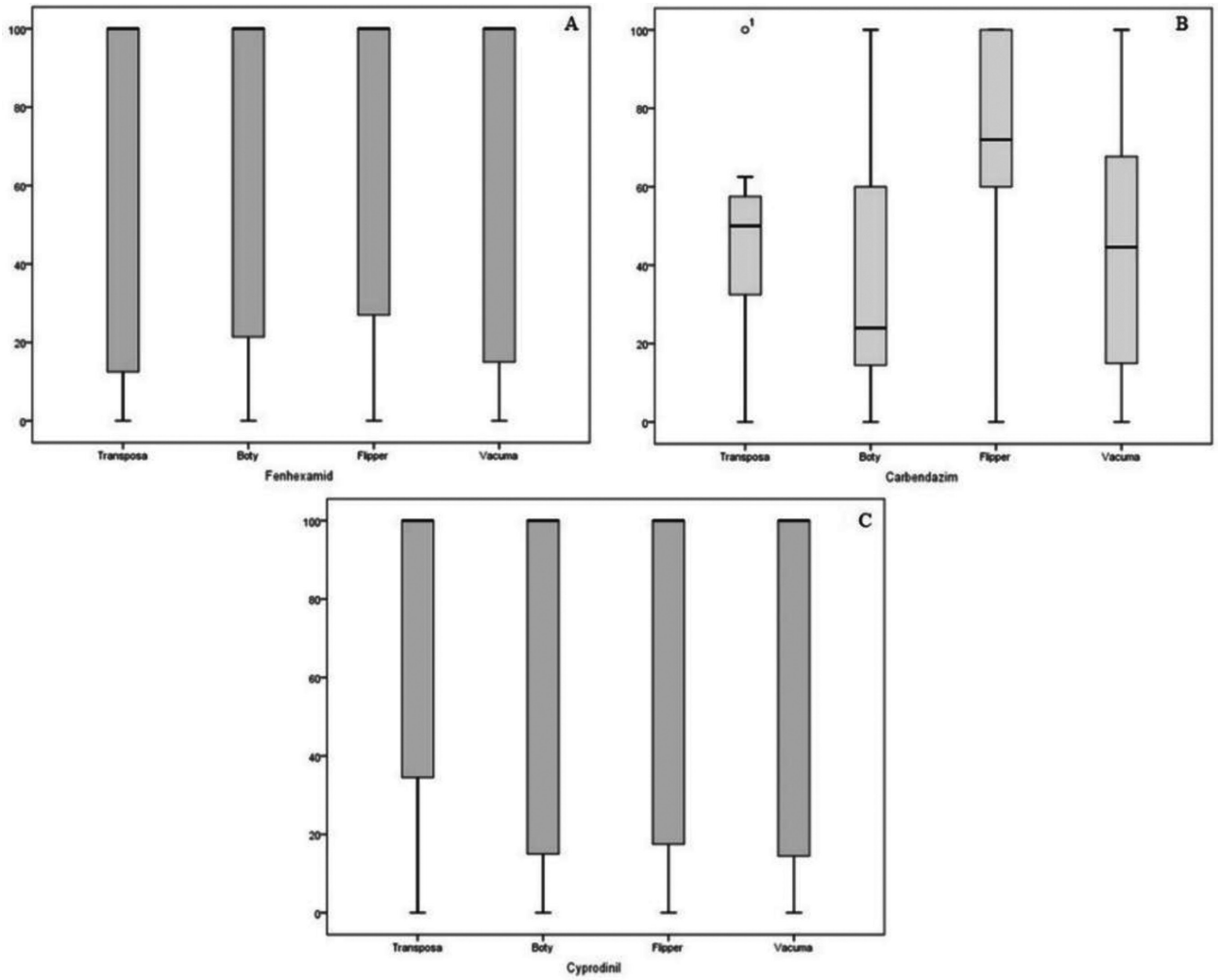

Figure 6. GIP value graphic of fungucides for mycelium stage tests. A: Fenhexamid, B: Carbendazim, C: Cyprodinil.

the high toxicity of fungicide; usage in higher doses is not recommended and suggested doses are not as effective as they are in laboratory conditions. *In-vitro* studies showed *B.cinerea* was sensitive to fenhexamid (IC_{50} lower than 6 mg/L), resistant to Carbendazim (IC_{50} greater than 50 mg/L), and tolerant to Cyprodinil (IC_{50} between 6-50 mg/L) [35]. Olea et al [36] studied antifungal effect of eugenol derivatives against *Botrytis cinerea* and found IC_{50} values between 31-95 ppm. Our results were ranged between 5.21-211.34 ppm depending on the fungicide tested. Comparing the transposon groups, for fenhexamid, *flipper* has the lowest resistance (27% inhibition), *transposa* has the highest resistance (12.5% inhibition). For carbendazim *flipper* has the lowest resistance (72% inhibition) while *boty* has the highest resistance (24% inhibition). For cyprodinil *transposa* has lowest reistance (40% inhibition) while *boty* has the highest resistance (17% inhibition).

Differences between spore and mycelium stage tests can be a result of different effect mechanisms of the fungicides. Among transposon groups *flipper* seems to be the most sensitive to fungicides thus found less in numbers. Although *boty* were more abundant, *B.cinerea* populations can change their transposon structure quickly, in one agricultural season different transposon profiles can be seen.

CONCLUSION

Fungicides play a major role in agriculture. It's a cost-effective method to fight diseases but excessive use harms consumers. Products can't be exported if fungicide residues are above the limits which results in economic loss. With each use there is also a risk for antifungal resistance development.

Table 7. Mycelium stage fungicide GIP test results.

Mycelium stage GIP values				
Fenhexamid GIP for mycelium tests				
Cons.	**Transposa**	**Boty**	**Flipper**	**Vacuma**
5000 mg/L	100	100	100	100
500 mg/L	100	100	100	100
50 mg/L	100	100	100	100
25 mg/L	100	100	100	100
12.5 mg/L	100	100	100	100
10 mg/L	100	100	100	100
5 mg/L	12.5	21.4	27	15
2 mg/L	10	20	20	5.6
0 mg/L	0	0	0	0
Carbendazim GIP for mycelium tests				
Cons.	**Transposa**	**Boty**	**Flipper**	**Vacuma**
1250 mg/L	100	100	100	100
1000 mg/L	62.5	62	100	70.7
750 mg/L	57.5	60	100	67.7
75 mg/L	52.5	27.4	100	51.8
50 mg/L	50	24	72	44.6
7.5 mg/L	47.5	20	65	33.8
5 mg/L	32.5	14.5	60	15
2.5 mg/L	25	12.9	51	13
0 mg/L	0	0	0	0
Cyprodinil GIP for mycelium tests				
Cons.	**Transposa**	**Boty**	**Flipper**	**Vacuma**
400 mg/L	100	100	100	100
200 mg/L	100	100	100	100
100 mg/L	100	100	100	100
50 mg/L	100	100	100	100
25 mg/L	100	100	100	100
20 mg/L	40	17	23	19
10 mg/L	29	13	12	10
0 mg/L	0	0	0	0

Table 8. IC_{50} values of transposon groups

IC_{50} Values				
Fenhexamid				
	Transposa	**Boty**	**Flipper**	**Vacuma**
Minimum	10	20	20	5.6
Maximum	100	100	100	100
IC_{50}	5.35	5.39	5.23	5.21
Hill Coefficient	52.29	53.16	50.42	52.91
Carbendazim				
Transposa	**Boty**	**Flipper**	**Vacuma**	
Minimum	25	12.9	51	13
Maximum	100	100	100	100
IC_{50}	165.63	166.1	51.99	211.34
Hill Coefficient	0.072	1.47	18.94	0.088
Cyprodinil				
	Transposa	**Boty**	**Flipper**	**Vacuma**
Minimum	29	13	12	10
Maximum	100	100	100	100
IC_{50}	20.39	20.39	20.24	20.27
Hill Coefficient	153.20	156.88	161.1	162.52

Although transposon profiling doesn't seem to have much practical use in short term, it can help farmers to change their choice of fungicide during season. This could reduce excessive uses of same fungicide thus reducing the antifungal resistance development.

In long-term, data can be obtained and a statistical data pool can be formed which will help to build better fighting strategies against the pathogen and fungicide resistance. A field survey can be made before planting and farmers can be advised on which fungicides to use and to decrease the doses used.

NOMENCLATURE

AC_{t0} Initial absorbance of control group
AF_{t0} Initial absorbance of fungicide treatment group
AC_{t2} Final absorbance of control group
AC_{t2} Final absorbance of fungicide treatment group

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AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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