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REGULAR ARTICLE

IN VITRO APPRAISAL OF INTERACTIONS BETWEEN SPOT BLOTCH PATHOGEN *B. SOROKINIANA* AND SELECTED PARASITIC FUNGI OF WHEAT LEAVES

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SUMMARY

Pathogenic micro-organisms living on wheat foliages may interact with each others. This study was conducted to reveal the interactive relationships existing between spot blotch causing fungus *Bipolaris sorokiniana* and selected pathogenic fungi subsisting on wheat foliages. Four fungal pathogens of wheat were selected, and the intensity and severity of the selected pathogenic fungi on wheat leaves were assessed. Pure cultures of the fungi were produced by isolating them from the spot blotch infected and blighted wheat leaves. Separate in vitro dual culture studies in completely randomized design with five replications were carried out to assess the interactions between each pair of B. sorokiniana and selected rival pathogens of wheat foliages. Percent inhibition in radial growth of either fungus was calculated. Viability test of the mycelium at the interface zone and pathogenicity test of the isolates were carried out. *B. sorokiniana* strongly inhibited the colony growth of *Cercospora* sp. and *Phoma* sp. under in vitro conditions. Similarly, there was not any effect on colony growth of either *B. sorokiniana* or *Bipolaris* sp. due to dual culture with each other. The dual culture of *B. sorokiniana* and *Alternaria triticina* results in the suppression of colony growth of both fungi. There were non-significant differences in percent growth inhibition between the first and the second week of dual culture in some of the tested fungi. The viability of mycelia of all the tested fungi was intact in dual culture. *B. sorokiniana* exerts antagonistic ability against some minor pathogens of wheat foliages under in-vitro conditions.

Key words: Antagonism, *Bipolaris sorokiniana*, Interactions, Pathogenic fungi, Wheat

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1. Introduction

Bipolaris sorokiniana (Shoem.), the causal agent of spot blotch disease, is the main biotic constraint of wheat in South Asia including Nepal (Dubin and Vanginkel, 1991). The disease severely affects more than 10 million hectares of wheat cultivation in Indo-Gangetic plains (Nagarajan and Kumar, 1998), and reduces grain yield up to 25% in the affected areas (Saari, 1997). Use of resistant genotypes and foliar sprays with fungicides are beneficial (Bhatta *et al*., 1997; Naitao and Yousan, 1997); however, lack of durable resistant genotypes and economic fungicides hinders management of the Spot blotch disease in South Asia.

Foremost pathogenic fungi inhabiting on various parts of wheat crop in major wheat growing areas of South Asia were reported by Dubin and Van Ginkel (1991). Few early reports revealed the presence of diverse interactions between pathogenic fungi of wheat phylloplane (Van-der wall *et al*., 1970; Brokenshire, 1974; Hyde, 1978). Studies on interactions among pathogenic micro-organisms of wheat phylloplane are being continued, but most of the works concentrate around the fungi which are not a major problem of South Asia. Neutral interactions were reported between wheat pathogens *Septoria nodorum* and *Septoria tritici* (Jenkins and Jones, 1981), and between *Pyrenophora tritici repentis* and *Septoria nodorum* (Da-luz,

1986). Very few interaction studies between *B. sorokiniana* and other fungi of wheat phylloplane have been concluded so far. Either neutral or antagonistic relationships were revealed between *B. sorokiniana* and *S. nodorum* (Da-luz, 1986). Antagonistic relationship between *B. sorokiniana* and Tan spot pathogen *Pyrenophora tritici repentis* was also identified under both *in vitro* (Ghazanfari, 1983) and at field conditions (Da-luz and Bergstrome, 1987).

Spot blotch disease has been a main concern of wheat research in South Asia. Interactions between spot blotch pathogen *B. sorokiniana* and other pathogenic fungi growing together on the same leaf may influence the development of one or both diseases. The knowledge on prevailing interactions between *B. sorokiniana* and other pathogenic fungi of wheat leaves will be useful in planning of resistant breeding strategy and development of biological and other control measures for spot blotch as well as the other emerging diseases of wheat. Therefore, this study was conducted to partially depict the existing interactions between *B. sorokiniana* and some pathogenic fungi of wheat foliage in the most hot spot location of spot blotch disease.

2. Materials and Methods

Prevalence and isolation of selected parasites

The experiment was conducted at the laboratory of National Wheat Research Program (NWRP), Bhairahawa, Nepal during February to June, 2009. Two-hundred spot blotch infected and blighted wheat leaf samples were randomly collected from different wheat genotypes grown at NWRP, Bhairahawa. The fresh leaf samples were kept in Petri plates containing three-layered moistened filter papers. The Petri plates were then incubated at 25±10 Celsius temperature for seven days. Four parasitic fungi were selected from the list in which major foliar pathogens of wheat in South Asia were reported by Dubin and Van Ginkel (1991). The intensity (frequency in tested samples) and severity (density of parasites on each sample) of the selected parasites on tested wheat samples were observed and recorded.

Isolations of selected parasitic fungi were carried out to prepare pure cultures of each fungus. Cut pieces of infected and blighted leaf samples were kept in Petri plates containing three-layered moistened filter papers. The Petri plates were then incubated at 25±10 Celsius temperature for seven days. Single spore isolation method was used for fungi having large size of spores, and frequent plate transfers method was used for fungi having smaller size of spores.

Identification of the selected parasites

The identification of selected parasitic fungi was carried out at the Department of Plant Pathology, Institute of Agriculture and Animal Science, Tribhuvan University, Chitwan, Nepal. The morphological characteristics of conidia/spores, conidiophores, fruiting bodies and colonies on PDA were studied and matched with identification catalogs.

Dual culture of *B. sorokiniana* and the selected parasites

Separate in vitro dual culture studies as suggested by Morton and Stroube (1955) were carried out to assess the interactions between each pair of selected parasites and *B. sorokiniana*. Each study having four treatments was conducted in Completely Randomized Design (CRD) with five replications. The treatments were contender parasite in dual culture with *B. sorokiniana*, contender parasite (sole culture), *B. sorokiniana* in dual culture with contender parasite, and *B. sorokiniana* (sole culture).

Mycelial plugs of 7 mm diameter taken from the growing edge of one week old pure cultures of both *B. sorokiniana* and the selected parasitic fungi were inoculated in Petri plates having PDA. The two plugs of the two organisms were placed with up side down position on opposite sides in a 90 mm diameter Petri dish at equal distance from each other and from the periphery.

All the inoculated Petri dishes were incubated at 25±10 Celsius temperature. The interactions between each pair of parasite and *B. sorokiniana* were studied and radial growths of *B. sorokiniana* and contender parasitic fungi were measured after seven

and 14 days of incubation. Percent inhibition in growth of *B. sorokiniana* and rival fungi was calculated using the following formula of Edington et al. (1971):

$$\text{Percent inhibition} = \frac{\text{colony radius of tested fungi (alone)} - \text{colony radius of fungi (with contender)}}{\text{colony radius of tested fungi (alone)}} \text{ X } 100$$

Interactions and viability of the mycelium

After 14 days of incubation, pieces of disc from the interfaced zone of each pair of dual culture were cut and transferred to a glass slide to examine under a microscope. The physical interactions between hypha of two fungi were also observed.

Ten mycelial discs (5 mm in diameter) of both fungi from the interfaced area of dual culture plates were taken after 14 days of incubation to examine the viability of mycelium. The discs were transferred to plane agar plates and incubated for ten days. The germination and growth of hypha and production of spores on each transferred sample were observed under a microscope after the incubation period.

Pathogenicity test

The pathogenicity of the *B. sorokiniana* and selected parasites of wheat leaves used in our study was tested in a poly-house to confirm their virulence. The experiment was arranged in a Complete Randomized Design with three replications. Five plants per pot were maintained for the test. Wheat seedlings were grown on polyethylene pots (17 cm in diameter and 15 cm in height) containing sterile soil. The seedlings were inoculated after 21 days of seeding (at three leaf stage) with cultures of *B. sorokiniana* and the selected pathogens at various spore concentrations. The concentrations of spores for pathogenicity test included *Bipolaris sorokiniana* [$1x10^4$ spores/ml (Duveiller and Altamirano, 2000)], *Bipolaris* species (specifera) [$1x10^5$ spores/ml (Han et al., 2003)], *Cercospora* species [$1x10^4$ spores/ml (Bargabus et al., 2004)], *Alternaria triticina* [$2 x10^5$ spores /ml (Pose et al., 2009)], and *Phoma* species [$8x10^6$ spores/ml (Sullivan and White, 2000)].

The poly-house was supplied with light irrigation for 48 hours to maintain high humidity. The seedlings were examined 14 days after inoculation for the development of typical symptoms of each pathogen. The leaf samples having typical symptoms were then incubated in moist chambers for seven days, and spores were isolated from the incubated leaf samples and observed under a microscope. The morphological characteristics were also studied and compared.

Data Analysis: The percent data were transferred in to arc sign values and analysis of variance for CRD performed. Normal colony growth data were directly analyzed using AOV for CRD. Means were compared using Fisher's Protected Least Significant Difference method (Steel and Torrie, 1980).

3. Results and Discussion

Prevalence of parasitic fungi on wheat foliages

The existence of selected parasitic fungi on wheat foliage is exhibited on Table 1. Most of the pathogens designated as wheat parasites by Dubin and Van Ginkel (1991) were present in the samples, which indicates that Bhairahawa conditions is very congenial for many foliar pathogens of wheat. Bhairahawa, Nepal has been identified as the most hot spot location of the spot blotch disease in South Asia (Joshi et al, 2007).

B. sorokiniana was very frequently isolated from mid to later stage of the crop with the highest frequency of 96 percent. The result signifies that spot blotch fungi continually and severely parasitize the wheat leaf after mid stage of the crop. Similarly, the frequency and intensity of *B. sorokiniana* on tested leaf samples were more than double as compared to the frequency and intensity of other pathogens. The result verifies that *B. sorokiniana* is the most dominant single pathogen of wheat foliages at Bhairahawa conditions (Table 1). Earlier reports advocated that Helminthosporium leaf blight (HLB) complex caused by a combined infections of *B. sorokiniana* and P. tritici repentis is the major leaf blight syndrome of wheat in plain areas of Nepal (Sharma et al., 2003).

The second most prevailed pathogen was P. tritici repentis, which was observed on 42 percent leaf samples. The frequency of P. tritici repentis in the tested samples was low, and was mostly isolated at the mid stage of the crop season. The result indicates that P. tritici repentis is not a severe problem at Bhairahawa conditions. Furthermore, the results disagree with the previous findings of Sharma et al. (2003) who reported that P. tritici repentis was abundant in HLB complex in Nepal. The lower prevalence of P. tritici repentis obtained in this study indicates the decreasing intensity of HLB complex and increasing intensity of spot blotch disease on leaf blight symptoms of wheat in plain areas of Nepal.

The frequencies of parasitic fungi *Phoma* species, *Cercospora* species, *Bipolaris* species and *Alternaria triticina* were in between 20 and 35% of tested leaf samples. The severity of *Phoma* spp. was the lowest, and the severity and intensity of *Bipolaris* spp. was also low. The frequency (21%) of *A. triticina* was the least, which indicates that *A. triticina* is not a major component of leaf blight complex at Bhairahawa conditions.

The infection of *Cercospora* spp. (28.5%) was mostly on late maturing varieties at later stage of the crop season. The prevalence of *Cercospora* spp. was mainly concentrated on a single month (March) with moderate severity. This is the first report of existence of *Cercospora* sp. as a foliar parasite of wheat at Nepal. The result indicates the arising problem of *Cercospora* leaf spot disease on later stage of the crop at Bhairahawa conditions.

Table 1. Major pathogenic fungi with their frequency on wheat leaf samples and intensity on single leaf at Bhairahawa, Nepal.

Name of the fungi	Frequency of fungi	Percent frequency	Mean severity on single leaf
Bipolaris sorokiniana	192	96.0	High
Pyrenophora tritici repentis	84	42.0	low
Phoma species	69	34.5	Very low
Cercospora species	57	28.5	Low to moderate
Bipolaris species	45	22.5	low
Alternaria triticina	42	21.0	low

Interactions between *B. sorokiniana* and *Cercospora* sp.

The radial growth of *Cercospora* sp. in dual culture with *B. sorokiniana* was significantly ($p<0.01$) lower as compared to that in the sole culture (Table 2). The radial growth of the fungi in different treatments is shown in Figure 1. The significantly lower radial growth of *Cercospora* sp. in dual culture indicates the strong antagonistic ability of *B. sorokiniana* against *Cercospora* species. Similar antagonistic ability of *B. sorokiniana* against wheat parasites *Pyrenophora tritici repentis* (Zhang and Pfender, 1992) and *Sclerotinia sclerotiorum* (Huang *et al*., 2000; Zhou and Reeleder, 1989) was also reported previously.

The infection of *Cercospora* sp. was observed on late maturing genotypes with low to moderate severity. The early and rapid infection of spot blotch disease with strong antagonistic ability of *B. sorokiniana* over *Cercospora* sp. might be some of the reasons of lower and late incidence of *Cercospora* leaf spot at Bhairahawa, Nepal. In addition, the suppression of *B. sorokiniana* through various management methods might enhance the epidemic of *Cercospora* species in future; however, the verification of the antagonistic relationship under field conditions must be needed before making such conclusions.

Table 2. Mean radial growth of *B. sorokiniana* and *Cercospora* sp. of wheat in an *in vitro* interaction study conducted at Bhairahawa, Nepal.

Pair of fungi in dual culture	Radius of fungal colony growth, mm			
	1st week		2nd week	
	B. sorokiniana	*Cercospora sp.*	*B. sorokiniana*	*Cercospora sp.*
Sole culture	15.80a†	12.70 a	21.80a	24.60 a
Dual culture	16.30a	08.30b	21.40a	09.00b
LSD ($P_{0.05}$)†	2.57	2.57	4.41	4.41
CV	14.5	14.5	17.1	17.1

† Means with in a column followed by different letters differ significantly based on LSD at p=0.05

Furthermore, the non significant (p<0.05) differences in radial growth of *B. sorokiniana* between its sole culture and dual culture with *Cercospora* sp. suggest the absence of any antagonistic effects of *Cercospora* species on *B. sorokiniana*.

The significant (p<0.01) antagonistic ability of *B. sorokiniana* over *Cercospora* sp. on both the 1st and 2nd weeks of dual culture suggests that the antagonistic ability is commenced at early growth periods and remained active through out the two-week interaction periods of this study.

Interactions between *B. sorokiniana* and *Bipolaris* sp.

There were non-significant differences (p<0.05) in radial growths of both *B. sorokiniana* and *Bipolaris* sp. in dual culture as compared to their sole cultures in the two week period (Table 3). The radial growth of the fungi in different treatments is shown in Figure 2. The non-significant difference in radial growth of both fungi indicates the presence of neutral interactions between the two pathogens in dual culture. These two pathogens belong to the same genus *Bipolaris*; therefore, could we suspect that there is neutral interaction between the pathogens of same genus?

Table 3. Mean radial growth of *B. sorokiniana* and *Bipolaris* sp. of wheat in an in vitro interaction study conducted at Bhairahawa, Nepal.

Pair of fungi in dual culture	Radius of fungal colony growth, mm			
	1st week		2nd week	
	B. sorokiniana	*Bipolaris* sp.	*B. sorokiniana*	*Bipolaris* sp.
Sole culture	17.40a†	10.20a	18.30a	11.00a
Dual culture	14.00a	7.00a	15.20a	7.80a
LSD ($P_{0.05}$)	3.547	3.547	3.601	3.601
CV	21.8	21.8	20.5	20.5

† Means with in a column followed by different letters differ significantly based on LSD at p=0.05

Our results do not agree with a similar report in which antagonistic ability of *Bipolaris* specifera against *Erysiphe polygoni* was identified (Suman, 2008). In contrast, Etebarian and Mohammadifar (2009) reported a strong antagonistic ability of popular biocontrol agents *Trichoderma harzianum* and *T. viride* against *Bipolaris specifera* under in vitro and micro plot conditions.

Interactions between *B. sorokiniana* and *Phoma* sp.

There was a significant (P<0.01) effect on radial growth of *Phoma* sp. due to dual culture with *B. sorokiniana* (Table 4). The radial growth of *Phoma* sp. and *B. sorokiniana* in different treatments is shown in Figure 3. The significantly lower radial growth of *Phoma* sp. in dual culture than in the control indicates strong antagonistic ability of *B. sorokiniana* on *Phoma* sp. under *in vitro* conditions. *Phoma* sp. is reported as a minor pathogen of wheat in South Asia (Dubin and

Van Ginkel, 1991). The results indicate that the high prevalence along with antagonistic nature of *B. sorokiniana* might partially suppress the normal dwelling of *Phoma* sp. on wheat leaves under Bhairahawa conditions; however, the *in vitro* antagonistic ability can not be equally effective under *in vivo* conditions (Baturo, 2006).

Table 4. Mean radial growth of *B. sorokiniana* and *Phoma* sp. of wheat in an *in vitro* interaction study conducted at Bhairahawa, Nepal.

Pair of fungi in dual culture	Radius of fungal colony growth, mm			
	1st week		2nd week	
	B. sorokiniana	*Phoma* sp.	*B. sorokiniana*	*Phoma* sp.
Sole culture	16.20a†	30.20a	18.80a	42.60a
Dual culture	15.70a	17.40b	17.60a	18.60b
LSD ($P_{0.05}$)	1.82	1.82	4.27	4.27
CV	6.8	6.8	13.1	13.1

† Means with in a column followed by different letters differ significantly based on LSD at p=0.05

On the other hand, the non-significant difference in radial growth of *B. sorokiniana* on dual culture and on control plates indicates the lack of any antagonistic effects of *Phoma* sp. on *B. sorokiniana*. The result does not accord with the previous findings in which the antagonistic ability of some *Phoma* species has been reported. For example, *Phoma glomerata* had antagonistic ability against powdery mildew parasite in oak (Sullivan and White, 2000), and an isolate of *Phoma* sp. from zoysia grass rhizosphere had suppressed *B. sorokiniana* by massive root colonization (Shivanna *et al.*, 1996). However, such findings could not be denied, because different species of a fungi and/or different strains of a species may have different level of antagonism against another organism (Ozlem and Gary, 2003).

Interactions between *B. sorokiniana* and *Alternaria triticina*

There were significant inhibitions ($p<0.01$) in mycelial growth of both *Alternaria triticina and B. sorokiniana* due to interactions with each other in dual culture as compared to their sole cultures (Table 5). The radial growth of both fungi in different treatments is shown in Figure 4. The significantly ($p<0.01$) lower radial growth of both fungi on dual culture indicates the presence of mutual antagonism between the two fungi. The significant mutual antagonism between the two fungi during both 1st and 2nd weeks suggests that the mutual antagonistic ability is initiated at early growth periods and remained active until later stage of the growth.

Table 5. Mean radial growth of *B. sorokiniana* and *A. triticina* of wheat in an *in vitro* interaction study conducted at Bhairahawa, Nepal.

Pair of fungi in dual culture	Radius of fungal colony growth, mm			
	1st week		2nd week	
	B. sorokiniana	*A. triticina*	*B. sorokiniana*	*A. triticina*
Sole culture	15.40a†	19.60a	18.60a	28.40a
Dual culture	11.90b	14.10b	11.90b	17.00b
LSD ($P_{0.05}$)	1.63	1.63	3.58	3.58
CV	8.0	8.0	14.1	14.1

† Means with in a column followed by different letters differ significantly based on LSD at p=0.05

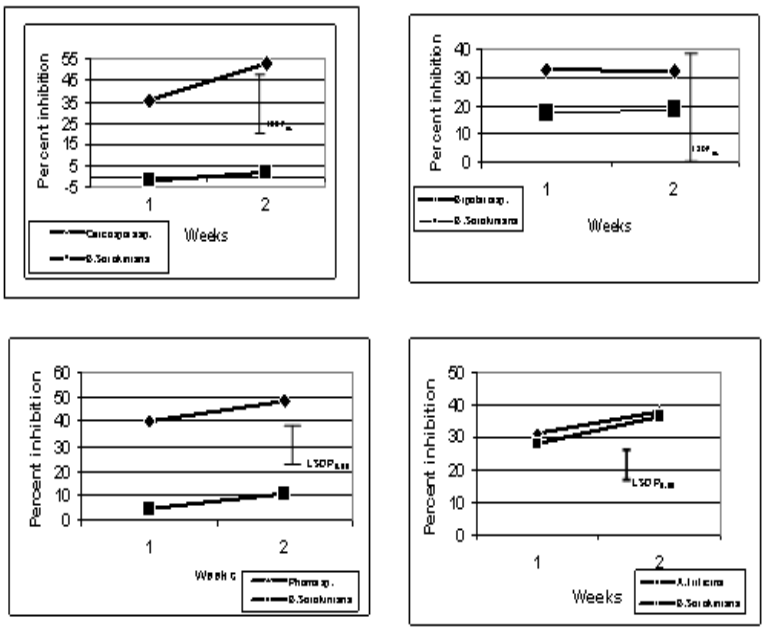
The results support the findings of some previous workers, who identified the antagonistic abilities of *Alternaria* species against a few plant pathogens (Liggett *et al.*, 1997; Musetti *et al.*, 2006). Similarly, the antagonistic ability of some fungi against *Alternaria species* has also been reported. For example, antagonistic ability of *Fusarium* sp. against *Alternaria raphani* in radish (Vannacci and Harman, 1987) and of *Epicoccum*

purpurascens, Aureobasidium pullulans and *Cladosporium cladosporoides* against *Alternaria brassicae* were reported (Rai and Singh, 1980).

Percent growth inhibition and ages of colony

There were non significant (P<0.05) differences in percent growth inhibition between one- and two- week old colonies of all the tested parasites in the dual culture with *B. sorokiniana* (Figure 5). The results suggest that the extent of inhibitory effects of *B. sorokiniana* on these fungi remain same through out the interaction periods.

Figure 5. Mean percent growth inhibition of selected saprophytic fungi in the 1st and 2nd week of interaction in *in vitro* studies conducted at Bhairahawa, Nepal.



In contrast, the percent growth inhibition (PGI) exerted by *A. triticina* on *B. sorokiniana* was significantly (P<0.05) higher on the 2nd week of dual culture as compared to the PGI of the 1st week. However, there were non significant (P<0.05) differences in percent growth inhibition between one- and two-week old colonies of *B. sorokiniana* in dual culture with the other three parasitic fungi (Figure 5). The significant differences in PGI of *B. sorokiniana* suggest that the extent of inhibitory effect of *A. triticina* on *B. sorokiniana* differs in different ages of the fungi. In addition, the higher PGI on the 2nd week as compared to the 1st week indicates that the antagonistic ability of the fungus is enhanced at matured stages.

Microscopic observation and viability of hypha

The colonies of both fungi of each pair did not touch to each other until the 14th day in dual culture. The hypha of tested fungi at the interfaced zone in dual culture was normal, and no physical contact of hypha of one fungus by another was observed under a microscope. The mycelial viability of all the tested fungi was intact at the interfaced zone in dual culture. The mechanisms of inhibition on growth of the tested fungi were not addressed in our studies; however, the significant inhibition in growth without direct contact of mycelia suggests that the prevailed antagonisms might be due to the production of inhibitory substances by the fungi or due to the competition for nutrients or both (Nourozian *et al.*, 2006).

Pathogenicity of the selected parasites

The typical symptoms of each pathogen were initiated on inoculated wheat leaves at 7-10 days after inoculations. Incubation of the wheat leaves having typical spots of *A. triticina* and *Bipolaris sp.* produced spores with in 7 days in moist chambers. Similarly, incubation of the wheat leaves having initial symptoms of *Cercospora* sp. and *Phoma* sp. produced typical fruiting structure and spores of each pathogen. The cultures of each of the pathogen on PDA produced typical colonies, fruiting structures and spores within three to four weeks of incubation.

4. Conclusions

Spot blotch fungus *B. sorokiniana* interacted differently with some parasites of wheat leaves under *in vitro* conditions. *B. sorokiniana* had strong antagonistic ability against *Cercospora* sp. and *Phoma* sp. In contrast, *B.* sorokiniana and *Bipolaris* sp. had neutral relationship with each other. Likewise, *B. sorokiniana* had mutual antagonism with *Alternaria triticina*. One of the reasons of lower intensity of minor pathogens *Cercospora* sp. and *Phoma* sp. in wheat foliage might be the strong antagonistic ability of *B. sorokiniana;* therefore the management schemes of *B. sorokiniana* must address the probable enhancement of the minor pathogens. However, the study on modes of action and antagonistic relationships of these fungi under field conditions must be conducted for constructive exploitation of the prevailed interactions.

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