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Bacterial and Fungal Pathogen Synergetics after Co-infection in the Wheat (*Triticum aestivum* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author MSD, SGW, PRB and KP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SAT and AAD managed the analyses of the study. Author MBP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Wheat is one of the most important staple grains in the world and the leading source of calories, production is limited by biotic stress. There is a number of pathogen attacks on wheat crops, depending on environmental conditions. In some cases, more than one crop pathogen attack leads to higher damage or decrease susceptibility. There are very few studies in the field of multiple pathogen interactions; in this study, we analyzed the co-infectionof wheat with fungal and bacterial pathogens. Field isolated *Xanthomonas translucens* and *Xanthomonas compestris* bacteria have been used against GM-322 and PDKV varieties co-infected with Fusarium fungus spp. In our experiment, we used *Fusarium oxysporum, Fusarium graminearum, Fusarium equitus*. Compared to the combined effect of the fungus and bacteria, we measured the length and width of the infected

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leaf part. We have observed that there is more susceptibility to *X. compestris* and *F. graminearium* in the GM-322 wheat variety. The second susceptible cvs was PDKV when we co-infected *F. oxysporum* and then *X. translucens* fungal and bacterial infected symptom analysis showed yellow stripes on the leaf surface of the wheat crop. We observed head blight in wheat when it was infected with *F. graminearum* and *X. compestris*. As a result, we concluded that varietal susceptibility also depends on co-infection pathogen attacks and their synergetic interaction.

Keywords: Wheat; pathogen interaction; Xanthomonas translucens; Xanthomonas compestris; Fusarium oxysporum; Fusarium graminearum.

1. INTRODUCTION

Wheat is one of the world's most important staple grains and is the leading source of calories and plant-derived protein in human food [1]. Wheat is primarily a rabi (winter) crop, and production is highly concentrated in the northern belt of Utter Pradesh, Punjab and Haryana, which together contribute 67% of total production and 55% of area [2,3]. Nevertheless, future production must increase as the global population is estimated to exceed nine billion people by 2050. As such, annual cereal production is expected to grow by nearly one billion tons. In addition, increased consumption of wheat products in many Asian countries and changes in grain quality requirements to meet the hidden hunger targets require additional crop production [4]. Many efforts are already underway in plant research in different parts of the world, but they are not only limited to improving crop production in crops such as wheat and other cereals, but also focused on basic plant science research with other experimental plants to develop methods that can then be applied to cereal crops with the ultimate aim of improving crop production [5-14]. In India, wheat is affected by various diseases such as rusts, powdery mild, loose smut, leaf blight, and kernal blunt [15]. Amongst these, kernal blunt is the major disease of wheat and caused by the fungus,*Tilletia indica* (Syn *Nevovosia indica*). It is the limiting factor in increasing wheat yield [16,17,18,19]. It affects mainly common wheat, durum wheat, triticale and other related species. The disease reduces seed quality, changes the chemical composition of infected grains and makes seed inedible. Wheat containing 3% bunted grains is unfit for human consumption [20].

Blast is one of the most devastating diseases of wheat. It is commonly referred to as leaf blast, collar rot node blast, spike blast or rotten neck blast depending on the portion of the infected wheat. The disease is also known to occur in triticale, barley and black oats. *Fusarium* head blight, root rot and foot rot (crown rot) are diseases that cause significant yield losses in a number of crops worldwide, such as wheat, maize, oat (*Avena sativa L.*) Rice (*Oryza sativa L*) [21,22]. The losses caused by Bacterial leaf streak as 40 percent have occurred in the most severely diseased field in Idaho, United states, although losses are generally 10 percent or less [23]. Typically, symptoms on the leaf consist of elongated, light brown lesions, several centimeters long, which are initially distinct but later coalesce to cover larger solid areas. Early symptoms are characterized by translucent stripes that are easily seen under incident light. Initially, lesions are water-soaked and produce honey like exudates giving a milky slime under humid condition *X. translucens pv.* Undulosa grows fastest *in vitro* at 28°C to 30°C. The bacterium can be cultivated on common medium, such as nutrient agar and Wilbrinks medium [24]. These culture media are not semi-selective and can be used in a wide range of bacteria. Since the bacterial leaf streak was first identified on wheat, a number of names have been used for X. Translucence strain isolated from small grains, however, these strains have not always been subject to differential host range tests for pathogenicity. The pathogen is non-sporic, rod-shaped, gram-negative and single-polar flagellum motile. *X. translucens* is oxidative and there is no nitratereduction in nitrite. We further analyzed the co-infection of these bacteria and fungi to study their interactions [25,26,27].

2. MATERIALS AND METHODS

2.1 Collection of Infected Leaves and Isolation of Bacteria

The infected field was analyzed for disease symptoms and the infected plant sample was collected by a small cut of the leaf tissue from the edge of the lesion. Crush the sample in sterile water with the help of forceps and scalpel. Small aliquots were produced from this sample and used to grow on plates, and the plates were

incubated upside down at 28°C for 1-3 days and analyzed continuously. Pure bacterial culture was obtained by further subculture and then characterized by gram staining and maintained by the preparation of slants.

2.2 Fungal Infection

Varieties of wheat seeds are planted in Petri plates, and after one week the seeds are planted in pots as control plants. Then we prepare PDA media and grow fungus on it, such as *F. oxysporum* (F.o), *F. graminearum* (F.g) and *F. equities* (F.eq), incubation time of *Fusarium* fungus growth is 4 to 5 days at 27°C incubator. Take 10 ml of autoclave distilled water in the test tube and then add the *Fusarium* fungus suspension. Inoculate the seed in the *Fusarium* suspension by deepening it with the help of forceps. The inoculated seed was stored in Petri plates for 4 to 5 days. Then the seeds were transplanted into pots and the pots were labeled with the name of the variety. Fungal infection methodology previously described in Wagh *et al.* 2016a [28].

2.3 Bacterial Infection

The suspension of *X. translucens* and *X. campestris* strain was spread to the peptone agar medium and then cultivated in the dark inside the chamber at 28°C for 48 hours. The bacteria were collected by adding sterilized ultrapure water and the concentration of the bacteria was adjusted to 0.3 OD at 600 nm. For inoculation of wheat with *X. translucens, X.campestris* detached leaves were wounded with a needle and the wounded regions were then covered with bacterial suspension. In addition, the bacterial suspension (20 µl) containing 0.05 per cent of Tween 20 fell to the same wounded areas of the leaf surface at a higher inoculation rate. After inoculation, the samples leave w after 5 days, 50% of the leaf part showed symp

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toms of bacterial leaf blight. Increased length and width of the lesions with the help of Vernier Caliper, three lesions from three individual leaves were analyzed in three replicates. Bacterial infection methodology previously described in Wagh et al. 2016a [28].

3. RESULTS

3.1 Isolation of Bacteria

First, the leaf spike rust infected wheat disease sample was collected from Gandheli in the Aurangabad region as shown in (Fig.1), followed by characterization and symptom analysis of the infected sample. We observed that it is a bacterial *Xanthomonas.* Disease, after which we isolated the pathogen causing the nutrient agar media as shown in (Fig. 2, A and C). Two types of colonies were observed after 24 hours in white and yellow colonies. Isolated colonies under a microscope are observed by gram staining, as shown in (Fig. 2, B and D) *Xanthomonas* is a gram-negative rod-shaped bacteria.

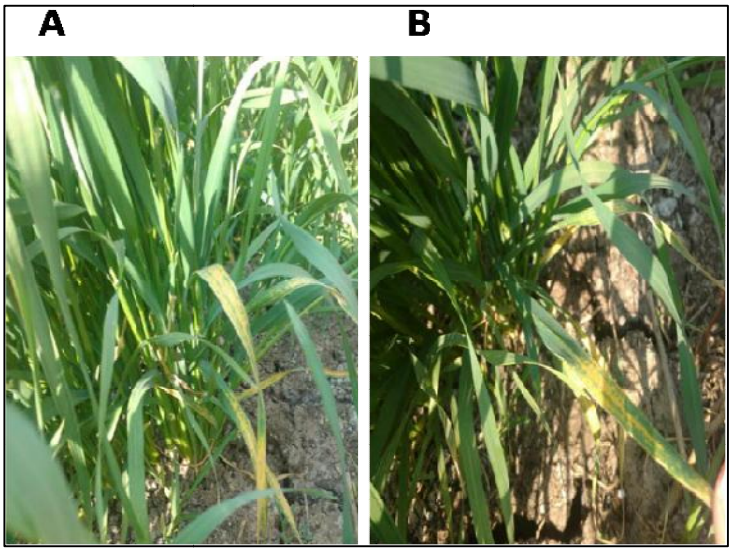

Fig. 1. Disease infected wheat sample

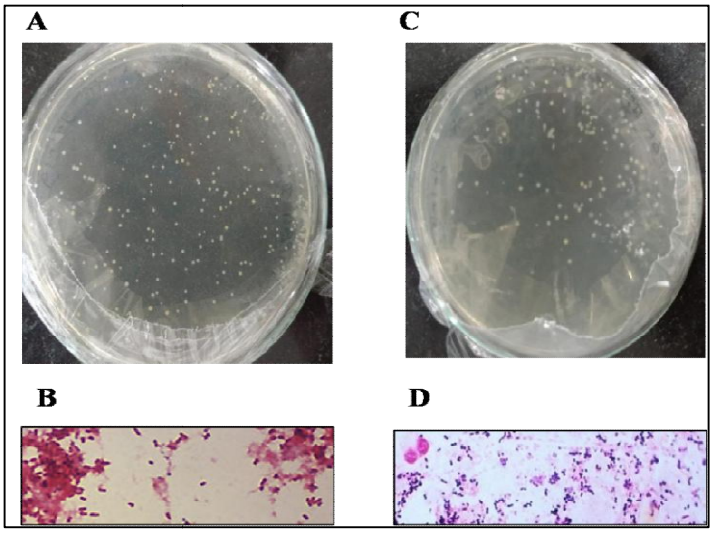

Fig. 2. *Xanthomonas* growing on nutrient agar (A and C) And gram staining (B and D)

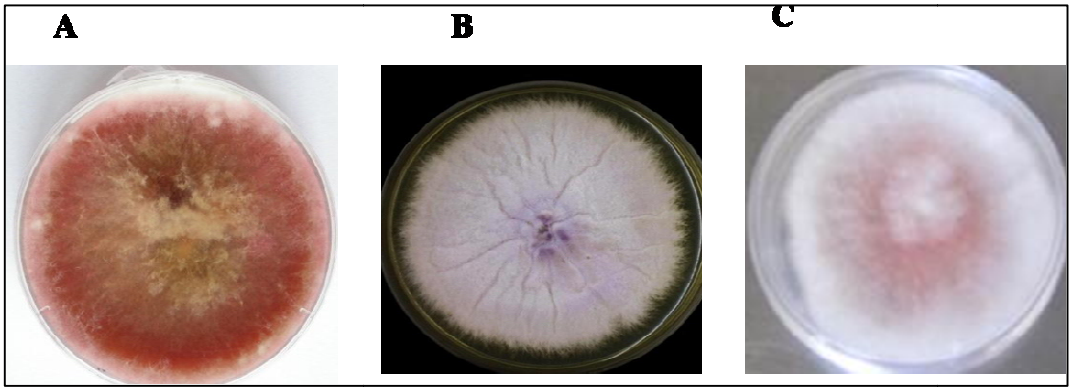

Fig. 3. From left A.*Fusarium graminearum, B. Fusarium oxysporum and C. Fusarium equitus* was grown on PDA media on plates

3.2 Preparation of Fungus Culture

Fungus spp., such as *F. graminearum, F. oxysporum* and *F. equitus*, was grown in the PDA media on plates as shown in (Fig. 3) and incubated for 4 to 5 days at 27°C in the incubator.

3.3 Inoculation of Seed with Fungus Culture

The seed was inoculated with the fungus by deepening the loop in 10 ml of distilled water and the seeds were deep into the fungus water of various varieties of wheat, such as GM322, PDKV, *F. graminearum, F. oxysporum* and *F. equitus* (Fig. 3). These fungus inoculated seeds were transplanted into pots after 4 days and the symptoms analysis was performed after 30 to 40 days when the leaves were grown enough to be analyzed.

3.4 Fungal and Bacterial Infection to Leaf

First infection with the fungus occurred at the early stage of the seedling with *Fusarium spp*. Infected plant leaf we used for infection of *X. translucens* and *X. compestris* after 35 days and kept at 25°C. Vernier Caliper was performed after 4 days of symptom analysis and the result is shown in Fig. 4. By measuring length and width with the help of Vernier caliper, we have observed that the susceptibility is greater in the GM-322 type of wheat when we co-infected first with *F. graminearum* and then with *X. translucens* in the GM-322 type of wheat. The

length was 1.2 inch and 0.8, the width was measured as shown in the graph (Fig. 4). The second resistance variety was PDKV, the length and width measured with Vernier caliper and the second observation is that the susceptibility to PDKV is greater in the wheat variety when we co-infected *F. oxysporum* first and then *X. translucens* in the wheat variety PDKV. The length was 0.8 and the width was 0.6, as shown in the graph. (Fig. 4).

3.5 Symptom Analysis after 35 to 40 Days

After 35 to 40 days symptom analysis was carried out. Co-infection with *X. translucens* and *F. oxysporum* together it shows symptoms like yellow colour stripe on leaf surface of wheat crop as shown in (Fig. 5). It mean that fungus *F. oxysporum* and bacteria *X. translucens* shows the synergetic effect on wheat crop as shown in (Table 1).

Pathogen-Pathogen interaction in wheat, two pathogen *F. graminearum, Psudomonas flurescens* showed synergetic interaction as shown in (Table 1). Similar result we observed in our study, co-infection of *F. graminearum* and *X. translucens* showed synergistic effect as shown in (Table 1) and shows symptom of head blight in wheat as shown in (Fig. 5).

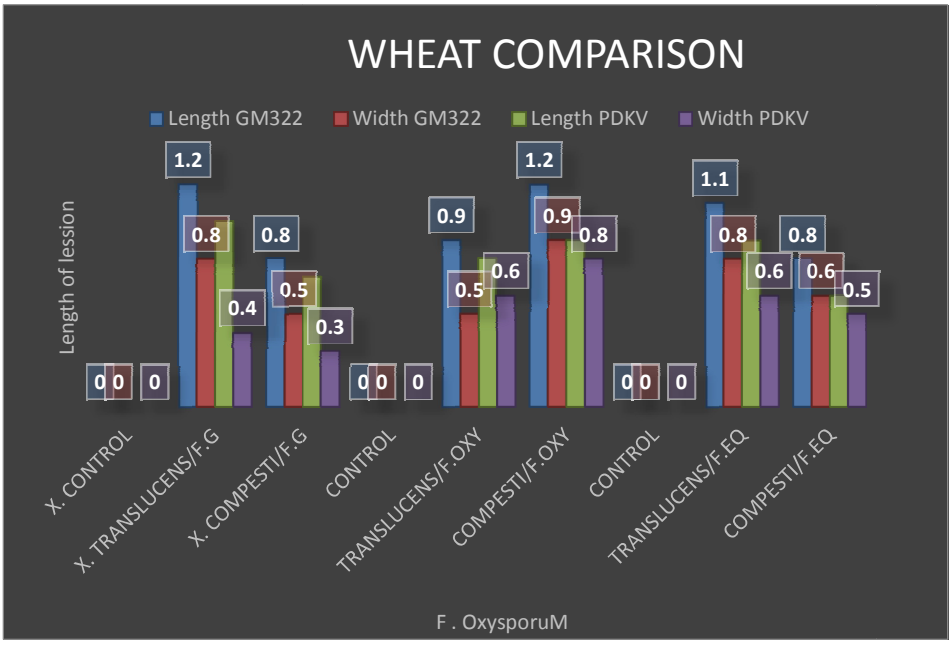

Fig. 4. Lesions length and width analysis in wheat genotypes

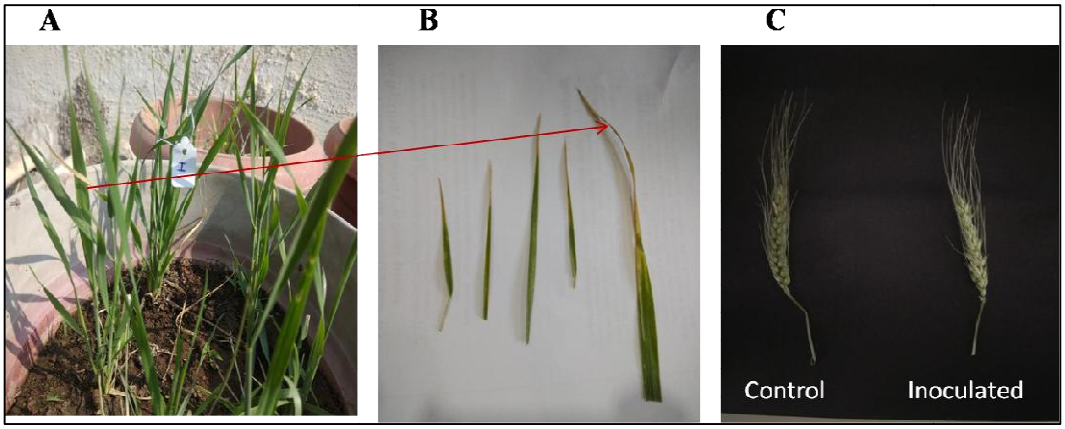

Fig. 5. Symptom analysis after 35 to 40 days

Table 1. Examples of pathogen-pathogen interaction in plant and pathogen species

Pathogen species.	Host	Co-infection type	Interaction type	References
Fusariumoxysporum/Psuedomonas flurescens	Wheat	Snchronous	Synergistic	Notz et al., 2002 [29]
Zymoeseptoria tritici/Blumeria graminis tritici	Wheat	Synchronus/ asynchronus	Antagonastic	Orton and Brown,2016 [30]
Fusarium oxysporum/ Xanthomonas translucens	Wheat	Synchronus	Synergistic	Our study
Fusarium graminearum/ Xanthomonas campestris	Wheat	Synchronus	Synergistic	Our study

4. DISCUSSION

Studies of plant–pathogen interactions have historically focused on simple models of infection involving single host-single disease systems. However, plant infections often involve multiple species and/or genotypes and exhibit complexities not captured in single host single disease systems. Here, pathogen interactions include: (i) Competition, in which competing pathogens develop physical barriers or utilize toxins to exclude competitors from resource-dense niches; (ii) Cooperation, whereby pathogens beneficially interact, by providing mutual biochemical signals essential for pathogenesis, or through functional complementation via the exchange of resources necessary for survival; (iii) Coexistence, whereby pathogens can stably coexist through niche specialization [31]. Furthermore, hosts are also able to, actively or passively; modulate niche competition through defense responses that target at least one pathogen [32,33]. Three key interactions can cause damage in co-infected plants: host– pathogen, pathogen–pathogen, and host-multiple-pathogen complexes. Host–pathogen interactions are well studied and are generally detrimental to the plant resulting in reduced fitness [24].

In contrast, pathogen–pathogen and host-multiple-pathogen interactions are less studied. These interactions can lead to various results: antagonism, synergism, coexistence, mutualism, or cooperation. The level of disease damage the plant experience varies depending on the outcome of the interactions and the corresponding host responses.

In our present study of multi pathogen host interaction in wheat, we observed that the effect arising from the fungus was not so much toxic than that, it means host pathogen interaction between F. spp and wheat crop is antagonstic. When we infect wheat crop with bacteria *X. translucens* and fungus *Fusarium spp.* Together, the together effect of bacteria and fungus is greater than that of the individual effect of Fusarium spp. It means host multiple pathogen interaction shows synergetic effect of multiple pathogen. When we co-infect *F. graminearum* and *X. translucens* together, we observed Fusarium head blight symptoms in wheat [32]. Pathogen-pathogen interaction in wheat plant two species co-infected *F. oxysporum* and P*suedomonas flurescens* it shows the synergetic type of interaction. Similar result we observed in our study when we co-infected *F. oxysporum*and *X. translucens* it showed the synergistic effect. *X. translucens* is wheat pathogens. Despite the demonstrated importance of bacterial species as potential donors of virulence genes in fungal cereal pathogens, currently limited genome sequence information is available for cereal infecting bacteria and this makers searching for new such genes difficult. Lesions in the infiltrated zone were observed after inoculations in this species. These lesions expanded over time and become water-soaked and necrotic, consistent wheat description of *X. translucens* pathogenicity on wheat.

In addition, bacterial concentration increases exponentially over time infiltrated wheat leaves, consistent with the virulent interaction on the species the pathogenicity of DAR61454 was also tested in over 100 wheat which all produce water soaked lesions inoculation this experiment indicated that *X. translucens*is highly pathogenic on wheat. F.g survival can be control by the enhancement of decomposition process. Agriculture practice are key factor for the control of F.g survival. A suitable crop rotation and an invasive tillage can limit the risk of Fusarium head blight development. The symptoms was seen in the infected area of wheat leaves, the black colour spots were obtained in that area in which bacteria (*X. compestris and X. translucens*) and fungus (*F.g, F.o & F.eq*) were

co-infected with the help of needle. Day by day the lesions were slowly developed from the first day to the fourth day, the complete development of lesion was 1.2 inches in length and 0.9 cm in width. And this length and width were measured with the help of Vernier caliper and data was collected. Future studies can be done by the use of CRISPER-CAS9 technology [13,14].

Our present study multi-pathogen-host interaction in wheat suggest that the susceptibility is more in the variety of GM-322 of wheat when we co-infect first *F. graminium* and then *X. translucens in* the variety GM322 of wheat. The length was 1.2 inch and 0.8 width was measured. The second susceptible variety was PDKV, the length and width measure with help of Vernier caliper.The second observation is that the susceptibility is more in the variety of PDKV of wheat, when we first co-infect *F. oxysporum* and then *X. translucens* in the variety of wheat PDKV, in contrast with Abdullah, et al. 2014 [33]. The length was 0.8 and 0.6 was its width. When we co-infect *F. oxysporum* and then *X. compestris* in the variety PDKV the length was 0.9 and 0.8 was its width. After 35 to 40 days symptoms analysis was done for co- infection with bacteria and fungus spp. Which shows the yellow colour stripe on leaf surface. Co-infection with *F. graminearum* and *X. compestris* shows the symptoms head blight in wheat, the co-infection can be reason of forming new deadly disease combinations [35]. Such interactions can be further studied in different types of cultivars and different envorinations can be used for the development of new pathogen resistant cultivars, with RNAi and CRISPER technologies [36,37]. These combinations can be taken into consideration for further breeding perspective.

5. CONCLUSION

The synergism observed between *F. oxysporum / X. translucens* and *F.graminearum / X. compestris* leads to increased disease susceptibility of both pathogens, increased damage to plant physiology and productivity. Hence, we concluded that varietal susceptibility is also dependent on co-infection pathogen attacks and interaction. These combinations can also be taken into consideration for further breeding perspective.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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