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ANTI-USTILAGO TRITICI POTENTIAL OF AQUEOUS EXTRACTS OF SELECTED PLANT SPECIES AGAINST LOOSE SMUT OF WHEAT

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Abstract

Triticum aestivum (L) is grown as an important food source around the globe and its demand is increasing day by day with the increasing human population. But its cultivation is becoming challenging and difficult due to the attack of *Ustilago tritici* due to which great yield losses are reported worldwide. Utilization of chemical-based fungicides against *U. tritici* is expensive with hazardous impact on human health while local farmers are unaware of the use of resistant varieties, which is also increasing the potential threat of fungal spread to non-resistant varieties. Loose smut of wheat is a devastating disease of wheat which spreads due to attack of *U. tritici* which common seed is born disease. Present experimental study was split into two consecutive years 2019-2021. Fungal strains were isolated during first growing season from Morocco (W1) the most susceptible wheat variety. While Galaxy 2013 (W2) was infected with stereo microscopically identified teliospores of U. tritici which were isolated during the first growing season. Plant extracts of *Azadirachta indica* L. (AIE), *Nicotiana tabacum* L (NTE), *Eugenia jambolana* L (EJE) and *Eucalyptus globules* L. (EGE) were applied as an experimental organic fungicide by keeping Tebucanazole DS as positive control in a Completely Randomized Block Design (CRBD). Statistically analyzed results showed that disease severity of *U. tritici* was lowest by AIE (12%), as compared to untreated plot (100%). While disease control was only seen among AIE and NTE (88 and 82) % after CBF (100 % disease control). Therefore, both of these plant extracts are recommended for the isolation of its active ingredient for the synthesis of safe alternative to chemical-based fungicides.

Keywords: Biotrophic, pathogen, smut, wheat, phytotoxicity

INTRODUCTION

Wheat (*Triticum aestivum* L) is among essential cereal crops which are cultivated largely around the globe after rice (27%) and maize (25%), which accounts 30% of financial records worldwide. This crop has attained the great importance and attention of farmers as well researcher due to be a vital source of energy and protein for human diet. This crop is basic source of diet for human especially for the citizens of a country like Pakistan, India and Bangladesh (Arshad and Shafqat 2012). No doubt wheat crop is cultivated on large scale in most countries it had been estimated that 600 million tons (MT) of wheat is grown around the world covering an area of almost 2-5 million hectares.

The contribution of underdeveloped countries is 55% in the total production of wheat worldwide. It had been reported that the cultivation of this food crop in Pakistan is 25.09 million tons (MT) which covers the area of 8.8 million hectares annually. Value and the

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importance of this crop could not be denied and ignored due to the fact that this crop is contributing about 20% world's food calories. Wheat crop is susceptible to numerous devastating diseases such as rust and smut which causes enormous damages and yield losses. Loose smut of wheat is infectious wheat disease which is caused by species of *U. tritici*, a biotrophic fungi. This disease is common among cereal crops and flourishes during the historic time, which were recognized by Roman people who designated it Ustilago a word from Latin meaning burns (Saari, 1996; Gad et al., 2019) (Ahmed et al., 2022).

U. tritici is a fungal pathogen which can cause loose smut of wheat which is commonly considered as a threatening plant disease which causes 40% of yield losses (Quijano et al., 2016). It had been a great threat for the production of seeds especially for developing countries which is a major concern for the land owners of small lands. Who are just cultivating wheat to meet their food requirements and uses seeds which were stored last growing season? Loose smut of wheat flourish more if found favorable environmental conditions such as cool and humid climate at a thesis stage of crop therefore, the chances for the losses could be significant in the regions having favorable environmental conditions for fungal spread. But losses due to this disease are also reported in some other regions of dry and warm climate as well (Carolina et al., 2016). The chances for the attack of this disease are between early and mid-anthesis stages of crop but successful infection can occur even after anthesis. Attack of this disease is easily recognized physically at the emergence of ear as each grain becomes completely or partially replaced by a mass of blackish fungal spores. These fungal spores released after the emergence of ear which leaving bare remains of the ear rachis behind, which is the clear indication of disease severity. Spores of these pathogens spread passively at the growing points of crop and develops into a smutted ear (Malik and Batts, 1960). The problem with the infected seed is that it never showed any kind of sign or mark which is obvious or unambiguous until the appearance of infected ears on crop (Eibel et al., 2005; Abrahim, 2019).

The only recommended solution to overcome this fungal pathogen is the use of healthy seed material of resistant varieties. Cultivation of resistant varieties are most effective measures in controlling the disease which is preventive measure, while chemical treatment is the protection method. There are numerous other chemical-based fungicides which are commonly available in the market to treat this disease. The use of chemical based fungicidal material to control these pathogens is a common practice which had great adverse results on the environment so for that’s why an alternative ways and tools are needed to overcome the use of chemical-based material to improve cropping system (Badar et al., 2020; Badar et al., 2022).

Both protective and preventive management strategies for loose smut of wheat are not effective for farmers cultivating wheat al low scale for their own. Cultivation of resistant varieties for local farmer grown wheat for meeting their food requirements is not in practice as they are using seeds stored from their previous year crops. Moreover, they are not familiar with the advance and resistant varieties. The use of chemical-based

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fungicide is not affordable for small scale producers and have numerous toxic effects on environment. Therefore, present experimental investigation was designed for the evaluation of economic and environmentally friendly plant based fungicidal material.

METHODS

Selection of most susceptible wheat variety

The current research work was performed in two consecutive years from 2019 to 2021. In the first year, screening of most susceptible wheat variety was done and the fungal isolates were isolated for further study. In the next year, the most susceptible wheat variety was grown and anti-Ustillago tritici potential of different phytoextracts was investigated for that variety. For this purpose, the seeds of four wheat varieties, namely Morocco (W1), Galaxy 2013 (W2), Ghazi 2008 (W3) and Akbar 2019 (W4) were purchased from Punjab Seeds Cooperation Sahiwal, Pakistan and cultivated in four different plots in Randomized Complete Block Design (RCBD) in the area of Kotmoman, District Sargodha, and Punjab, Pakistan.

Collection and preservation

Teliospores (Sample RS-01) from freshly smutted spikes of wheat had been collected from affected spikes of wheat crop (Morocco). These teliospores were shade dried, gently scraped thoroughly by 53µm mesh sieve, sealed in pre-labelled cellophane plastic bags which were refrigerated at 10°C. Potential of these strains were tested using water agar and those shown viability of >70% were further used (Nasr, 1977; Rajput et al., 2019).

Identification of teliospores

Plant infected spikes were photographed by digiporo-Labomed (PX 5) and illustrated with the help of photographs taken from microscope attached camera in the field. While, free-hand sections were made and mount of infected parts were observed under a Labomed CSM2 stereomicroscope. At least 30-35 spores of each spore stage were examined under a compound microscope (MX4300H, Meiji Techo Co., Ltd., Japan) for spore measurement. Each spore stage was photographed (Gupta et al., 2009)

Molecular identification

Molecular identification of the collected teliospores were carried by Pablo Alvarado Garcia Spain, while the amplification of Internal Transcribed Spacer Region (ITS) has been done via couple ITS-specific primers (ITS1 forward and ITS4 reverse) (Table 1). The obtained sequences were BLAST on NCBI for sequence similarity with already identified strain of *U. tritici*. Genetic association and phylogenetic analysis have been done by neighbor-joining of multiple isolates of local *U. tritici*, which has been compared with multiple sequences of nitrogenous bases by depending on ITS interface region (Al-Yassiry and Al-Alwani 2022).

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Table 1: Primers for molecular identification

Primer name	Primer sequence (5'-3')	rRNA operon binding site
ITS1F (F)	CTTGGTCATTTAGAGGAAGTAA	Small subunit
ITS4 (R)	TCCTCCGCTTATTGATATGC	Large subunit

ITS1F= forward primer, ITS4=reverse primer, F=forward, R= reverse and rRNA=Ribosomal Ribonucleic acid

Anti-*Ustilago tritici* activity experiments: In the next year, Galaxy 2013 was grown in six experimental plots (Table 2) in RCBD in greenhouse provided all favorable conditions for fungal growth (temperature below 25 ^{o}C) (Saari et al., 1996; Gad et al., 2019) for the evaluation of fungicidal potential of experimental material. Seeds were treated 24 hours before sowing with all experimental formulations (10, 20 and 50) % of all the four plants extracts and Tebuconazole DS was also applied at three concentration (10, 20 and 50) % while untreated crop was served as check.

Table 2: Experimental design during second growing season (2021-2022)

Plant extracts	Dosage (%)	Replications		
		R1	R2	R3
T1	C1	T1C1R1	T1C1R2	T1C1R3
	C2	T1C2R1	T1C2R2	T1C2R3
	C3	T1C3R1	T1C3R2	T1C3R3
T2	C1	T2C1R1	T2C1R2	T2C1R3
	C2	T2C2R1	T2C2R2	T2C2R3
	C3	T2C3R1	T2C3R2	T2C3R3
T3	C1	T3C1R1	T3C1R2	T3C1R3
	C2	T3C2R1	T3C2R2	T3C2R3
	C3	T3C3R1	T3C3R2	T3C3R3
T4	C1	T4C1R1	T4C1R2	T4C1R3
	C2	T4C2R1	T4C2R2	T4C2R3
	C3	T4C3R1	T4C3R2	T4C3R3
T5	C1	T5C1R1	T5C1R2	T5C1R3
	C2	T5C2R1	T5C2R2	T5C2R3
	C3	T5C3R1	T5C3R2	T5C3R3
T6	N	T6NR1	T6NR2	T6NR3

In this table, T1 to T6 are the names of blocks. T1 stands for AIE (*Azadirachta indica* extracts); T2 for NTE (*Nicotiana tabacum* extracts), T3 for EJE (Eugenia jambolana extracts), T4 for EGE (*Eucalyptus globules* extracts), T5 CBF (*Tebuconazole* DS) and T6 for UNC (Un-Treated Crop). N stands for Negative Control. Out of all these plots, CBF and UNC were taken as positive and negative control respectively.

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Collection and processing of plant material

Leaves of *Azadirachta indica* L., *Nicotiana tabacum* L., *Eugenia jambolana* L. and Eucalyptus globules L. were collected from Pakistan Council of Scientific and Industrial Research (PCSIR), Lahore, Pakistan. In order to proceed the study further these samples were rinsed with tap water, air dried for 4 days and then refrigerated at 4°C till further use.

Preparation of fungal inoculum and phytoextracts

For homogenous spore suspension, 4g spores had been added in 1.0 L dist. H2O and few drops of Tween ® 20, followed by the adjustment of inoculum density to 4×l06 spores by hemocytometer (Nasr, 1977). Phyto-extracts of all plant leaves were prepared by converting the dried leaves samples in to fine powder (sieved through 80 mesh size). Ten grams of each powdered sample was extracted by 100 ml sterilized dist. H2O in 250 ml conical flask, left for 8 h at 25oC, filtered through ash less filter paper, followed by the preparation of stock solution 1:1(mg/ml) and stored in refrigerator at 4 °C for further use (Bashir et al., 2019).

Foliar Application

When *T. aestivum* L reached at anthesis stage, spikes were inoculated with inoculum suspension through atomizer by spraying method (Mishra et al., 1990; Navathe et al., 2020). Stock solution from each plant extract was used to prepare 10%, 20% and 50% solution, which were applied one day before and after 7, 14 and 21 days of artificial inoculation. Three different concentrations of Tebucanazole DS (10, 20 and 50) % as positive control (Wanyera et al., 2016; Kumar, 2020). The formula used for calculation of inhibition percentage

(IP) was IP= {(C-T)/C}*100

Estimation of Phytotoxicity

Phytotoxicity was estimated on the basis of physical observations (chlorosis, necrosis, stunting, wilting etc.) which were performed following guidelines by CIB using a reported rating scale of 0 to 10 (Muthukumar and Kumar 2015; Kumar 2020a and b).

Statistical Analysis

All the results and the collected data was statistically analyzed using Two Way Analysis of Variance (ANOVA) with the help of SPSS. All experiments were run in triplicate and antifungal efficacy of experimental materials was expressed as average ±SD of each fungicide at 5% level of significance (Steel et al., 1997).

RESULTS

Screening of susceptible wheat variety: All varieties of wheat showed blackish dry powdery mass on the spikelet which entirely replaced parts and glumes. Morocco was marked as the highly susceptible wheat variety (with 51-70% infected spike plants),

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followed by Galaxy 2013 and Ghazi (26-31% and 21-31% infected spikes. Respectively) while Akbar 2019 was found resistant due to least number of infected spikes (6-10%) (Table 3).

Table 3: Screening of susceptible wheat variety during the first crop season (2019-20)

Sr. No.	Wheat varieties	% of disease indecency	Symptoms
1.	Morocco	51-70%	HS
2.	Galaxy	26-31%	S
3.	Ghazi	21-31%	S
4.	Akbar 2019	6-10%	R

Four wheat verities were grown during 2019-2020 for the isolation of susceptible wheat variety. Here, HS stands for Highly Susceptible, S for Susceptible R for Resistant Pathogen microscopic identification: The teliospores were single celled, olivaceous brown to dark blackish brown, spherical to ovoid shaped, thick walled with slight reticulate ornamentation (Figure 1).

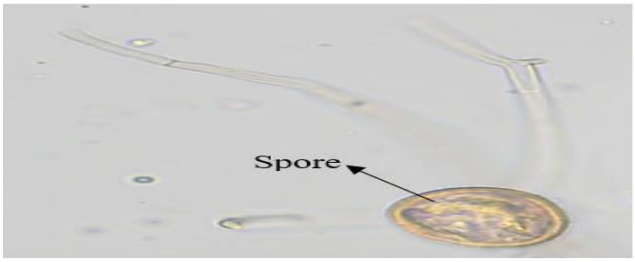

Fig 1: Microscopic image of Sample (RS-01) showing telial stage of *U. tritici* isolated from experimental crop

Molecular identification: Molecular analysis of the Sample (RS-01) suggested that it had a clear resemblance with the *U. tritici* (NCBI accession number is OP164708). It was further supported by the phylogenetic analysis, followed by the formulation of dendrogram from the available data (Figure 2).

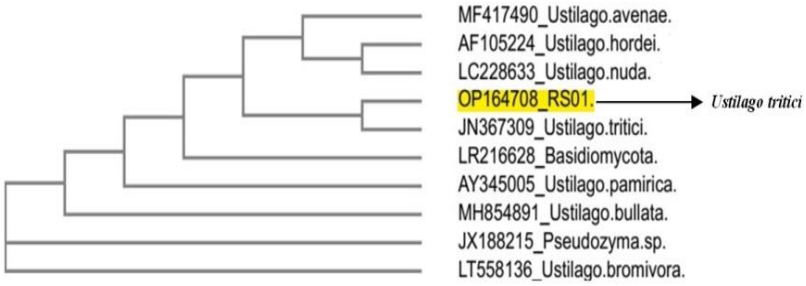

Fig 2: Phylogenetic tree of U. tritici on Minimum Evolution analysis, accession number of experimental Sample (RS-01) is highlighted, (OP164708_RS01). The obtained accession number for the isolated sample was used for the present course of study

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Antifungal efficacy of phytoextracts against *U. tritici*

The Analysis of Variance (ANOVA) reflected that there was no significant difference among all fungicides ($p>0.05$) including phytoextracts and chemical based commercially used fungicide. This result is extremely valuable as it reflected that the phytoextracts are effective at par with synthetic chemicals in controlling the growth of fungal pathogen. It was also interesting to note that there was no significant difference among different concentrations in controlling fungal growth ($p>0.05$) which reflects that all samples were equally effective in controlling the pathogen. But it was noteworthy that the interaction between concentration and treatments significant difference in action ($p<0.05$) which means that combination of few treatments and concentrations could prove effective in controlling the pathogen under investigation.

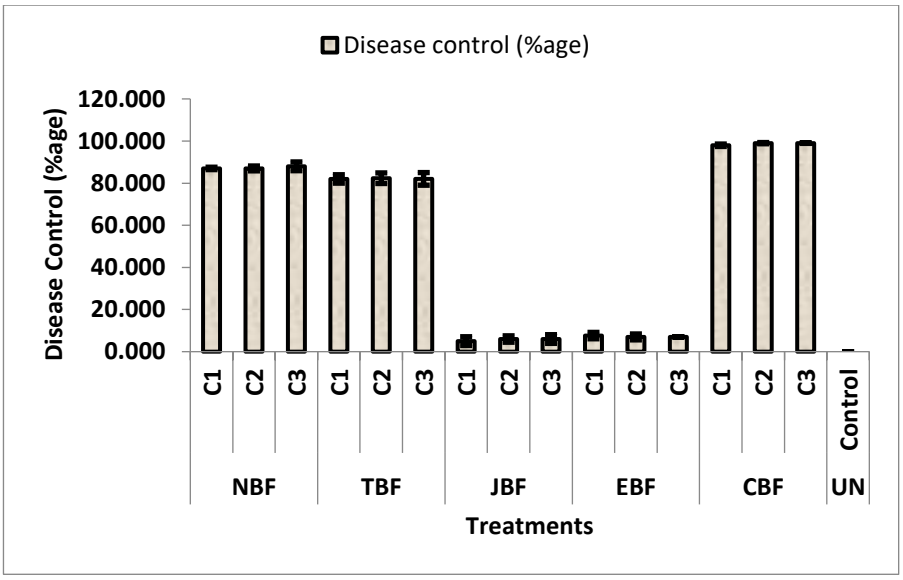

Fig. 3 Comparison of disease control by application of different treatments (n=3/treatment), where AIE (*Azadirachta indica* extracts, C1= 10%, C2=20% and C3=50%), NTE (Nicotiana tabacum extracts C1= 10%, C2=20% and C3=50%), EJE (*Eugenia jambolana* extracts C1= 10%, C2=20% and C3=50%), and EGE (*Eucalyptus globules* extracts C1= 10%, C2=20% and C3=50%), are the experimental treatments while CBF (Tebuconazole Chemical based fungicide) and UNC (Un-Treated Crop) were kept as control.

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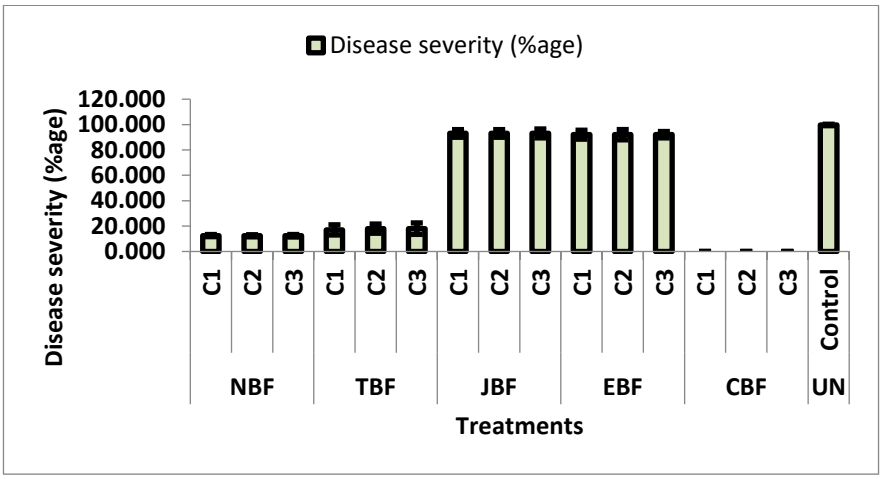

Fig 4: Comparison of disease severity by application of different treatments (n=3/treatment) calculated in percent (%). Results of experimental and control treatments are presented graphically which indicate the effectiveness of AIE and NTE. Where AIE (*Azadirachta indica* extracts, C1= 10%, C2=20% and C3=50%), NTE (*Nicotiana tabacum* extracts C1= 10%, C2=20% and C3=50%), EJE (*Eugenia jambolana* extracts C1= 10%, C2=20% and C3=50%), and EGE (*Eucalyptus globules* extracts C1=10%, C2=20% and C3=50%), are the experimental treatments while CBF (Tebuconazole Chemical based fungicide) and UNC (Un-Treated Crop) were kept as control.

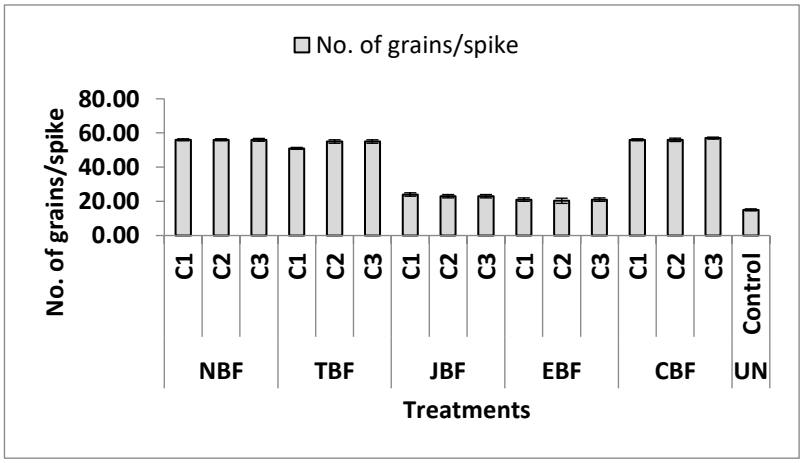

Fig 5: Illustration of the comparison of number of grains/spikes of Morocco after application of different treatments (n=3/treatment), experimental and control. While resulted outcomes of the study clearly showed the effectiveness of AIE and NTE. Where AIE (*Azadirachta indica* extracts, C1= 10%, C2=20% and C3=50%), NTE (*Nicotiana tabacum* extracts C1= 10%, C2=20% and C3=50%), EJE (*Eugenia jambolana* extracts C1= 10%, C2=20% and C3=50%), and EGE (*Eucalyptus globules* extracts C1= 10%, C2=20% and C3=50%), are the experimental treatments while CBF (Tebuconazole Chemical based fungicide) and UNC (Un-Treated Crop) were kept as control.

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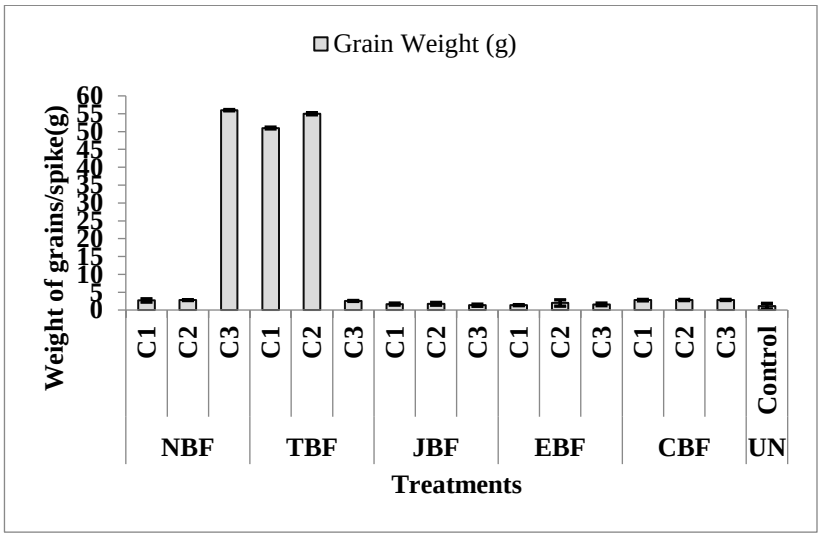

Fig 6: Comparison of weight of grains produced by Morocco after application of different treatments (n=3/treatment) for the calculation of disease control and disease severity. Where AIE (*Azadirachta indica* extracts, C1= 10%, C2=20% and C3=50%), NTE (*Nicotiana tabacum* extracts C1= 10%, C2=20% and C3=50%), EJE (*Eugenia jambolana* extracts C1= 10%, C2=20% and C3=50%), and EGE (*Eucalyptus globules* extracts C1= 10%, C2=20% and C3=50%), are the experimental treatments while CBF (Chemical based fungicide) and UNC (Un-Treated Crop) were kept as control.

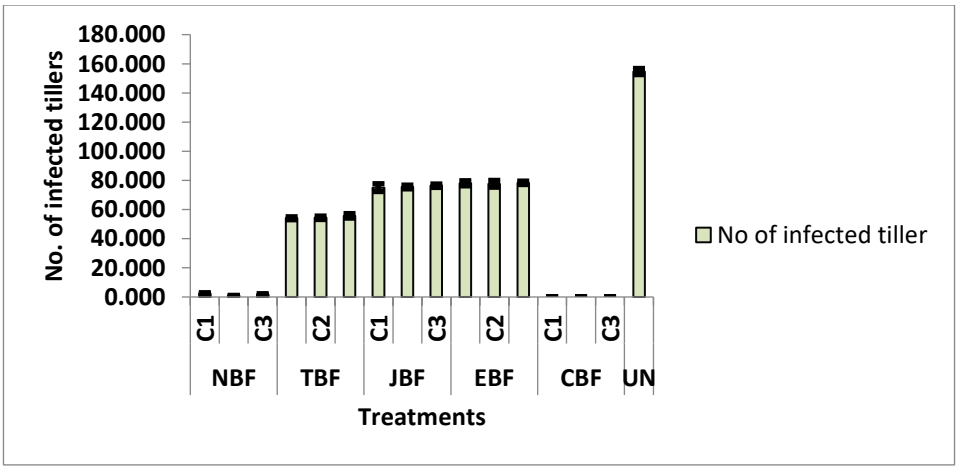

Fig.7 Comparison of number of tillers of Morocco produced after application of different treatments (n=3/treatment), indicating the effectiveness of treatments (experimental and control). Also highlighting the effect of these treatments on number tiller. Where AIE (*Azadirachta indica* extracts, C1= 10%, C2=20% and C3=50%), NTE (*Nicotiana tabacum* extracts C1= 10%, C2=20% and C3=50%), EJE (*Eugenia jambolana* extracts C1= 10%, C2=20% and C3=50%), and EGE (*Eucalyptus globules* extracts C1=

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10%, C2=20% and C3=50%), are the experimental treatments while CBF (Tebuconazole Chemical based fungicide) and UNC (Un-Treated Crop) were kept as control.

AIE were very effective in controlling loose smut of wheat when results were compared with the outcomes of CBF and untreated samples (UNC). Disease severity was highest in EJE followed by EGE (93% and 92 % respectively) with a greater number of infected tillers (76.20 & 79.0 respectively) and minimum number of grain/ spike (23.02 & 21.33 respectively), which yielded lowest grain weight/ spike in both of these experimental plots (1.50 & 1.43 respectively) as compared to CBF which showed 100% disease control (Figures 3-7). Therefore, the disease control was not recorded in UNC and highest number of fully or partially effected tillers were seen due to which there was lowest yield. Level of disease control in both plots (EJE and EGE) were lowest (6.01 and 7.89) % while disease control was seen 0% in untreated plot results are shown in Figure 3 & Figure 4. The disease severity was 12.0% in the plot treated with NTE followed by NTE (disease severity were found 18% and disease control were recorded 88% and 82%) and grain weight/ spike of 2.48 (gm) (Figures 3-7). It was evident that AIE has strong *U. tritici* activity at par with the synthetic chemical-based fungicide and it has the highest potential to be used as fungicidal material to overcome fungal crop enemies

Evaluation of Phytotoxicity

The experimentation performed to observe phytotoxicity symptoms confirmed that none of the organic material had shown phytotoxicity symptoms, thus strengthening the results of applied treatments.

DISCUSSION

Control of wheat fungus through plant extracts is an organic and alternative treatment to chemical method, which is economic and eco-friendly. Plants possess natural products due to which numerous plants such as *Azadirachta indica, Syzygium aromaticum, Nigella sativa, Schinus terebinthifolia, Allium sativum* and Garden quinine had been commonly known for inhibiting germination of the spores of wheat leaf rust caused by the attack of *P. triticina* by 93% or more (Shabana et al., 2017). Resulted outcomes of present study suggested that AIE had a great potential for the induction of plant resistance against loose smut cause by *U. tritici*. Results of present study were also favored by Karsou and Samara (2021) as they also reported plant products can act as inducers of resistance against wheat fungus. A. indica is a strong growth inhibitor for a wide variety of plant pathogens, parasites and pests. Antifungal potential of A. indica is also associated with the presence of phytochemicals such as phenolic, flavonoids and anti-oxidants (Badar et al., 2020; Badar et al., 2022). These phytochemicals are marked for the induction of defense mechanism against pathogens.

There are multiple defenses signaling pathways which are marked efficient for the induction of resistance in plants against pathogen activation of which is totally dependent upon the type of infection (Figure 5) (Aliferis et al., 2014). The mechanism of inhibition

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may be attributed to restriction of fungal germ tube growth through modification of cell walls by papilla formation. Defense mechanism in plants is associated with the PAMP (PTI) and effector-triggered immunity (ETI) to start the PRR mediated immune response through the production of ROS, which is an important protein responsible to induce pathogenesis (PR) along with the stimulation of secondary metabolites such as phenylalanine ammonia lyase, polyphenol oxidase, flavonoids etc, which act as signaling molecules and are the intermediates for systemic acquired resistance (SAR) against these disease causing agents (Kaur et al., 2022).

The findings of current research work are also supported by the concept of the induction of disease resistance among crops when treated with plant-based material by working as secondary messengers and by strengthening the host defense mechanism. The activation of host resistance through enhancing the activity of peroxidase (PO), along with the production of new (POD) isoforms followed by the accumulation of the phenolic compounds is yet another explanation of how these organic extracts can act in inhibiting fungal growth. The other reason for the positive outcomes of the study through the induction of disease resistance in host crop could be due to the antioxidative enzymes, which could enhance the production of high level of H2O2 (Karsou and Samara (2021) which retard the growth and survival of pathogen through disturbing the food supply to pathogen. This finding is supported by a study which confirmed that the production of H2O2 had a relation with the increased level of oxidative enzymes which is a great sign of host defense mechanism against disease causing pathogen (Motavallihaghi et al., 2022).

Polyphenol oxidase (tyrosinase) is also responsible for increased production of antioxidant enzymes which leads the expression of resistant in host crop (Ramzan et al., 2021). Peroxidases and polyphenol oxidases work synergistically as polyphenol oxidases enhance the activity of peroxidase through the production of phenolic compounds (Francoz et al., 2015). Most important reason for the development of plant resistance is by inactivation of pathogen enzymes by promoting the concentration of quinines in the host plant which results in unavailability of plant proteins to pathogens as nutrients. The increased level of superoxide dismutase (SOD) and peroxidase (POX) are responsible for resistance in wheat variety against bacterial blight (Fatemifard et al., 2022). Increased level of ROS in disease wheat had been also noted with more production of antioxidants (Chen et al., 2020). It is expected that current formulation has followed this mechanism to increase resistance in wheat crop.

Another study by Samanta et al. (2011) indicated that the presence of phenols, flavonoids and oxidative enzymes could possibly inhibit the growth of pathogens as concentration of these phytochemicals were higher in the roots of diseased plants (Samanta et al., 2011). Similar results of increased number of phytochemicals especially phenolic acids have been reported by Mathpal et al. (2016). It is noteworthy that the loos smut of wheat is seed borne disease, which does not appear just after the germination. The fungal pathogen forms a systemic infection while at the time of heading, it starts flourishing towards the head tissues and presents its symptoms. Similarly, findings of another study

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suggested that the high levels of phenol and some enzymes are the key components for the development of resistance in cereals against biotic stresses. In a recent study Rashad et al. (2018) confirmed a decline in infection by *Rizoctonia solani* with an increase in activity of poly phenol oxidases. Some enzymes are very important to serve as first line antioxidant defense such as SOD, CAT and GPX which had been reported to play an important part for the induction of the total defense mechanism in the biological systems (Ighodaro and Akinloye, 2018). Therefore, plant parts could be used to induce resistance against pathogenic organisms, which would be eco-friendly tool to inhibit these enemies.

CONCLUSION

It could be concluded that two out of the four (NBF and TBF) organic treatments had a great potential to be used as an effective fungicidal material against loose smut caused due to (*U. tritici* OP164708 _ RS01) of wheat (WVM). Therefore *A. indica* and *N. tabacum* based extracts are recommended for the organic and ecofriendly management against disease. As all the experimental material had shown no symptom of phytotoxicity due to which the chances for the safe and ecofriendly synthesis of antifungal material are more. Utilization of these plant parts for the synthesis of fungicidal material to control these enemies, with the increase of production is significant approach. Experimental extracts were found effective as were applied through foliar route at three different concentration 10%, 20% and 50% similarly formulations of same concentration for all treatments were applied as a seed treatment before sowing.

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Conflict of interests

There are no potential conflicts of interest declared by author (s).

Author contribution

RB performed all experimental work as a part of her PhD project and wrote 1st draft of manuscript and performed interpretation of results. AA designed and supervised the whole project, performed statistical analysis of the results, Edited, revised and gave final approval to this manuscript. SJ provided her technical support for the analysis of data and English editing services. SF provided her technical support for experimental work at PCSIR Lahore. FB provided her logistic support for a part of experimental work and technical support for the analysis of data. HW provided her English editing services and revised this manuscript, MM helped in write up and graphical form, give final form to the article, RM helped in review of literature and SH help in lab.

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