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Enhanced Systemic Resistance and Yield against *Puccinia triticina* Eriks in Wheat through Botanicals under *In-vivo* condition; An Eco-Friendly Approach

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ABSTRACT

In present study four types of botanical extracts including neem (*Azadirachta indica* L.), safeda (*Eucalyptus cameldulensis* Dehnh.), rosemary (*Rosmarinus officinalis* Spenn.) and ajwain (*Trachyspermum-ammi* L.) were selected and pre-inoculated on susceptible wheat cultivar "Morocco" to evaluate their effect on oxidative enzyme activity, chlorophyll contents, phenol contents and yield components against *Puccinia triticina* Eriks under *In-vivo* conditions during 2018/2019 growing seasons. Coefficient of infection (ACI) for all botanicals ranging between 6.50-16.50 as compared to control (ACI= 78.00) reflect the significant response in reducing infection of leaf rust. Ajwain was the most effective, its efficiency reached 91.66%, similar to the efficiency of fungicide spotless 12.5 WP (93.20%) followed by rosemary (85.25%), neem (82.00%) and safeda (78.84%). Moreover, all tested botanicals significantly increased the wheat yield components. Similarly, the biochemical analysis revealed that chlorophyll contents, oxidative enzymes activities (POX & PPO) and phenol contents in all treated plants increased. We conclude pre-application of botanical extracts is a promising approach for the management of leaf rust of wheat.

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INTRODUCTION

Leaf rust of wheat caused by the fungal pathogen *Puccinia triticina Eriks* is among the severer strains in wheat production and is widely distributed in several wheat growing regions of the world (Park *et al.,* 2007). In Pakistan this disease has appeared several times in an epidemic form (Chaudhry and Shah, 1996), causing severe damage to wheat crop and lowering the production of wheat. Under favorable environmental conditions, leaf rust pathogen alone can cause 30-50% yield losses. Previous history of rust epidemics indicated the vital impact of this disease (Luck *et al.*, 2011). Leaf rust of wheat is a long-standing threat to wheat production in wheat-growing regions around the world. Because of the high genetic diversity and widespread occurrence, leaf rust pathogen results in greater yield losses than stripe and stem rusts (Huerta-Espino *et al.,* 2011). Planting resistant cultivars is the basic way to reduce the disease (Draz *et al.,* 2015). Synthetic fungicides were also used to minimize the virulence of pathogen (Barro *et al.,* 2017). Beyond breeding for genetic resistance, large scale removal of alternative host and cultural practices can minimize pathogen diversity and spawn new races (Peterson, 2001). Rust Pathogen has the ability to form new races that are much more virulent and capable of breakdown the plant resistance, this is the big reason behind the epidemics of leaf rust in wheat. Application of fungicides is increasing every day, and this generates negative effects on environment. Thus, substitute approaches including, the use of botanicals and organisms are being established to reduce the use of chemicals against various pathogens. Researchers have adopted the use of botanical against a lethal pathogen because, many of them contain different types of antifungal substances with less influence on surrounding environment. Different plants contain different type of components which are toxic in nature and inhibit the prevalence of many pathogens, namely, as botanicals or botanical pesticides (Dubey *et al.,* 2008). From the past few decades' scientist have proved natural products as an antimicrobial agent and environmentally safe, which could help in controlling various types of pathogens and provide a leading way to plant protection (Wang *et al.,* 2004). In some susceptible plant's, resistance can be induced systemically by the application of different chemicals and can be induced by pre-inoculation of non-pathogenic or pathogenic micro-organisms (Kuc, 1982). According to Chakraborty and Chakraborty (2010) induced defense response is triggered by certain types of components in infected plants, through which botanical extracts effectively control the wide range of lethal pathogens (Shrivastava *et al.,* 2011). According to Morsy *et al.* (2011), studies on botanical extracts have gained much more attention from past few years. Abiotic inducers act as a secondary messenger (Bhuvaneshwari *et al.,* 2015), that enhance the host defense mechanism by increasing the production of (POD) peroxidase isozymes, or by increasing the activity of peroxidase (POX) (Hassan *et al.,* 2007; Geetha and Shetty, 2001) or through inhibition of some catalyst and antioxidant, that results into elevated amount of H_2O_2. Through direct effects abiotic inducers also increase resistance against the survival and development of pathogens or indirectly have subsequent effect on food supply of pathogens (Khan *et al.,* 2003; Radwan *et al.,* 2008). The present experiment was done to explore the potential of different botanical as inducers to enhance resistance against leaf rust pathogen under *In-vivo* conditions.

MATERIALS AND METHODS

Plant material

Four botanical extracts belonging to different species, together with leaves of neem (*Azadirachta indica L.*), safeda (*Eucalyptus cameldulensis*), rosemary (*Rosmarinus officinalis*) and ajwain (*Trachyspermum-ammi*) powder were used for the examination of their efficacy as inducers against leaf rust infection in fields located in the research area of Department of Plant Pathology, University of Agriculture Faisalabad. Healthy and fresh leaves of rosemary, safeda and neem were collected from 32 square experimental area of Horticulture Institute, University of Agriculture Faisalabad. Ajwain powder was obtained from the local market. Before extraction, all collected leaves specimens were cleaned and wipe by running water to remove the dust particles. After air drying all samples were kept at 4 °C in refrigerator till use.

Preparation of botanical extracts

All botanical extracts were rehearsed by using the technique explained by Hussain *et al.* (2012). Parts of the plant which are used in experiment were individually grinded to semi-powder form by using a grinder. 10 g of each grinded sample was taken in a conical flask along with 100 ml distilled water and kept at room temperature for 6 hours. Then the stock solution was filtered through filter paper (Whatman 1) to separate the solid particles. Whole procedure was repeated five times to obtain clear botanical extract. After that all the extracts were kept in refrigerator at 4 °C.

Application process of botanical extracts

The experimental area of Department of Plant Pathology, University of Agriculture Faisalabad, was used to conduct the whole experiment during two growing seasons (2018/2019). Seeds of susceptible cv. "Morocco" were obtained from Wheat Research Institute, Ayub Agriculture Research Institute (AARI). Experiment was laid out in Randomized complete block design (RCBD) with three replicates. During two growing seasons, seeds were sown in random plots at the rate of 50g/plot. Plant extracts were prepared at (10000 mg/l) and sprayed on the plant until wetness by using a hand sprayer. Chemical fungicide spotless 12.5 WP (difenoconazole) at the rate of 0.75 g/l was taken as control. Artificial inoculation was done at booting growth stage of plants by urediniospores of rust fungal isolates. According to (Lorrain *et al*., 2019), mixture was prepared by using talcum powder (20 volume) and one volume of urediniospores. Before inoculation plants were treated with water by using hand sprayer to maintain the relative humid conditions and then wiped with urediniospores of rust fungal. During sunset dusting of urediniospores was carried out. Inoculation was done at the 7-8th growth stage, during the second half of February (Draz *et al*., 2019). The untreated plots served as control. Disease was investigated by using the disease rating scale based on septicity types of leaf rust of wheat, explained by Johnston and Browder (1964), where immune (0) = no visible infection on plants, resistant (R) = visible chlorosis or necrosis no uredia are present, moderately resistant (MR) = small uredia are present that are either surrounded by chlorosis or necrosis, moderately susceptible (MS) = medium size uredia are present and possibly surrounded by chlorotic areas and susceptible (S) = large uredia are present on the surface of plant. Severity of leaf rust disease articulated as percentage exposure of leaves (Bock *et al.*, 2021). According to Saari and Wilcoxson (1974) average coefficient of infection (ACI) was calculated by multiplying constant values of infection types of viz. R = 0.2, MR = 0.4, MRMS = 0.6, MS = 0.8 and S = 1.0 and disease severity. Effectiveness of treatments was determined by using the equation (Rewal and Jhoty, 1985).

$$Efficiency\ \% = \frac{\mathrm{C} - \mathrm{T}}{C} \times 100$$

Where, C = represents the infection in control and T = represents the infection in treatment

Effect of all treatments were estimated on grain yield components (1000-kernel weight, spike weight and volume weight) at the time of harvest.

Biochemical assay

All the biochemical changes in inoculated and un-inoculated wheat plants were estimated at the High-tech Laboratory of University of Agriculture Faisalabad. Leaf samples of all treatments and controls were collected to measure their effects on chlorophyll, phenols and oxidative enzymes after 1^{st}, 7^{th} and 14^{th} days.

Chlorophyll contents

According to Dere *et al.* (1998) total chlorophyll (a + b) was determined through following procedure. Leaves of all treatments and control were amended into small pieces of size (1 mm). The cutting samples were immersed in 20 ml methanol for 24 h at 4 °C and filtered by using Whatman 47 mm filter paper. A blank of 96% methanol was used to measure the absorbance of each filtrate at wavelength of 666-653 nm. Results chlorophyll a & b were calculated and expressed in mg/g^{-1} by using the formulas developed by Linchtenthaler and Willburn, (1983), for Chlorophyll a = (15.65 A666 – 7.34 A653), for Chlorophyll b = (27.05 A653 – 11.21 A666) and total chlorophyll = Chlorophyll. a + Chlorophyll. B.

Phenol contents

Total phenol contents were measured by using following protocol described by Malick and Singh (1980). 10 ml 80% ethanol was used for 0.5 g of fresh leaves to ground the leaves and kept in a bottle for 72 h at 4 °C. Unico UV-2100 spectrophotometer was used to determine the phenol contents of filtered extracts. Folin-Ciocalteu's reagent was used to measure the total phenol contents and absorbance was read at 650 nm and expressed in mg g^{-1}.

Oxidative enzymes activity

By following the typical procedure explained by Hammerschmidt *et al.* (1982), peroxidase action can be easily estimated. Furthermore, activity of polyphenol oxidase was easily determined by using protocol explained by Malik and Singh (1980), in which 3 ml of 50 Mm TRIS buffer with (PH 7.8), containing 7.5% polyvinylpyrrolidone and 1 mM EDTA-Na2 was used to homogenize 0.5 g leaf material of each treatment and control at 0 - 4 °C. To measure the total enzymatic activity through spectrophotometer, homogenized mixture was centrifuged at 12,000 rpm for 20 min, by maintaining 4 °C temperature. All measurements were conceded by using spectrophotometer at 25 °C. To minimizes the error whole assays were performed three time. With various intervals of 30 s changes in absorbance for PPO at 495 and POX at 470 were recorded for 5 minutes. Activity of enzyme was expressed in (min^{-1} g^{-1}).

Statistical analysis

Collected data were imperiled to (ANOVA) by using statistics 8.1 and means were separated using (LSD) at $P \leq 0.05$ explained by (Steel and Torrie, 1980).

RESULTS

In table 1, effect of various botanical extracts on severity of rust pathogen were presented. Data showed that spraying of tested botanical on susceptible wheat plants of "Morocco" variety before infection had effective role in controlling the rust pathogen. Average coefficient of infection (ACI) was determined to check the effectiveness of plant extracts against disease incidence of leaf rust of wheat. It is observed that all plant extracts significantly reduced the disease incidence. ACI value for Ajwain was recorded (6.50) which was insignificantly different from fungicide treatment with ACI = 5.30. whereas average coefficient value for control plants increased and reached up to 78.00. Tested botanical extracts significantly reduced the incidence of leaf rust of wheat whereas the efficacy of all extracts ranged between 78.84 for Safeda and 91.66 for Ajwain extracts as given in (Table 1).

By keeping in view, the yield components, it was observed that all tested botanical extracts expressively increase the kernel weight, spike weight and volume weight of the wheat plants infected with rust pathogen as compared to control. Ajwain and rosemary gave best result in increasing the yield components. Ajwain and rosemary gave maximum spike weight (4.83 and 4.55 g respectively) and gave best results by increasing 1000 kernel weight and volume weight (56 and 53.23 g/ 824.31 and 818.11), respectively. While Safeda and Neem was proving to be least effective as compared to Ajwain and Rosemary. Results of yield attributes showed that Ajwain and Rosemary were the superlative treatments that increased the yield of infected plants in comparison with fungicide presented as followed.

Table 1. Average coefficient of infection (ACI) and yield components of wheat plants treated with various botanicals against *Puccinia triticina* Eriks under *In-vivo* conditions.

Effect of Treatments			Yield Components		
Treatments	ACI	Efficiency %	Spike weight (g)	1000-kernel weight (g)	Volume weight/L
Rosemary	11.50 cd	85.25	4.55 b	53.23 b	818.11 b
Safeda	16.50 c	78.84	4.44 b	50.24 c	805.82 d
Neem	14.00 cd	82	4.54 b	52.65 b	811.23 c
Ajwain	6.50 de	91.66	4.83 a	56.00 a	824.31 a
Spotless 12.5WP	5.30 e	93.2	4.55 b	54.40 ab	811.50 c
Control	78.00 a		3.85 d	45.03 d	739.80 f
LSD 0.05	6.86		0.25	1.99	3.2

Biochemical analysis of wheat leaves

Chlorophyll contents

Results of chlorophyll (a + b) contents illustrated that all tested botanicals gradually increased the chlorophyll contents of wheat plants up to 14th days after spraying as compared to control, in which chlorophyll contents (a + b) decreases after the same period (14 days). Ajwain extract was found to be most effective in increasing the chlorophyll contents of infected leaves of wheat after first, seventh and fourteenth days of spray with (3.3, 3.8 and 4.2 mg/g), followed by rosemary (3.2, 3.5 and 3.9 mg chlorophyll/g fresh weight) respectively as compared to control (2.5, 2.3 and 1.9 mg/g) and fungicide treatment (2.6, 3 and 3.6 mg/g) as given in (Figure 1).

Phenol contents

The data presented in Figure 2 showed the effect of different botanical extracts on phenol contents. It was estimated that all botanical extracts significantly increase the phenol contents of infected plants as compared to non-treated control. Phenol contents gradually increases after spraying with plant extracts. Ajwain extract found to be successful in increasing phenol contents (12, 23.1, 62 mg/g fresh weight) at 1, 3 and 14th days after spraying followed by rosemary (11, 21, 50 mg/g), neem (9, 19, 47 mg/g) and safeda (8, 17, 45 mg/g) respectively. Whereas the total phenol contents in control (5, 5 and 8 mg/g fresh weight) and fungicide (8, 11 and 30 mg/g) were recorded as showed in (Figure 2).

Oxidative enzymes activity

After spraying all the treatments, it was noticed that oxidative enzymes productivity enhanced on 1st and 3rd days as compared to control treatment showed in (Figure 3 and 4). Increased activity of enzymes (POX) peroxidase, (PPO) polyphenol oxidase were observed in all plants treated with plant extracts. Ajwain and rosemary were found to be effective in increasing the POX activity followed by neem and safeda which showed lowest efficacy during 1, 3 and 14-day periods of time as given in (Figure 3). Furthermore, it was also observed that PPO activity also increased in infected plants which are treated with plant extracts. Ajwain and Rosemary found to be most effective in increasing the PPO activity in infected wheat plants as compared to non-treated control and fungicide, while neem and safeda showed lowest efficacy.

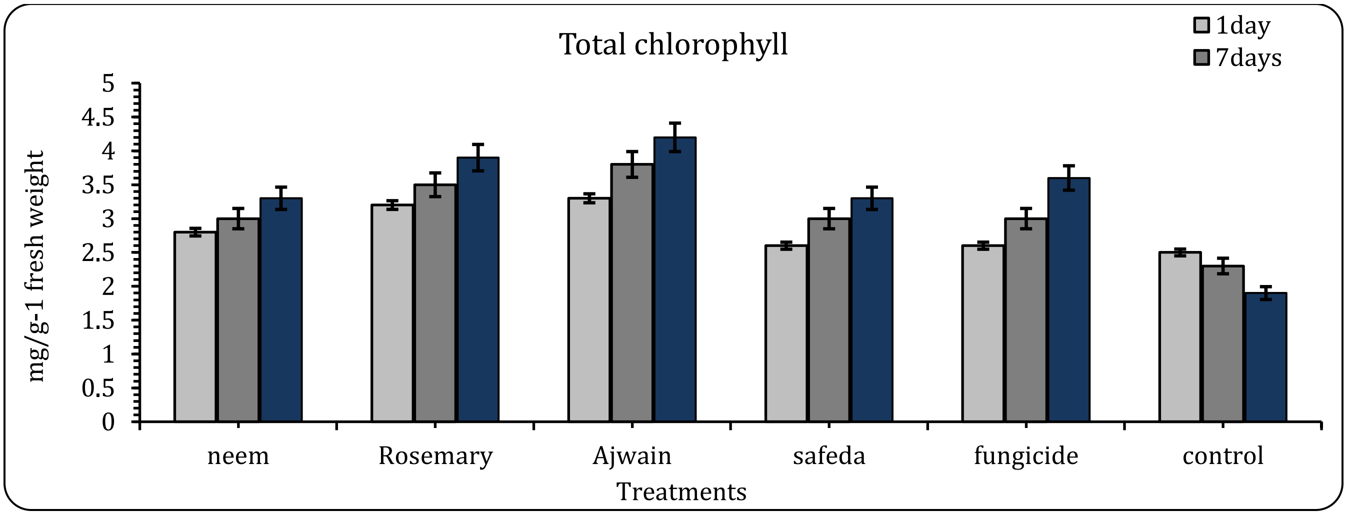

Figure 1. Comparison of total chlorophyll contents (a + b) of wheat leaves treated with different botanicals during infection of *Puccinia triticina* Eriks.

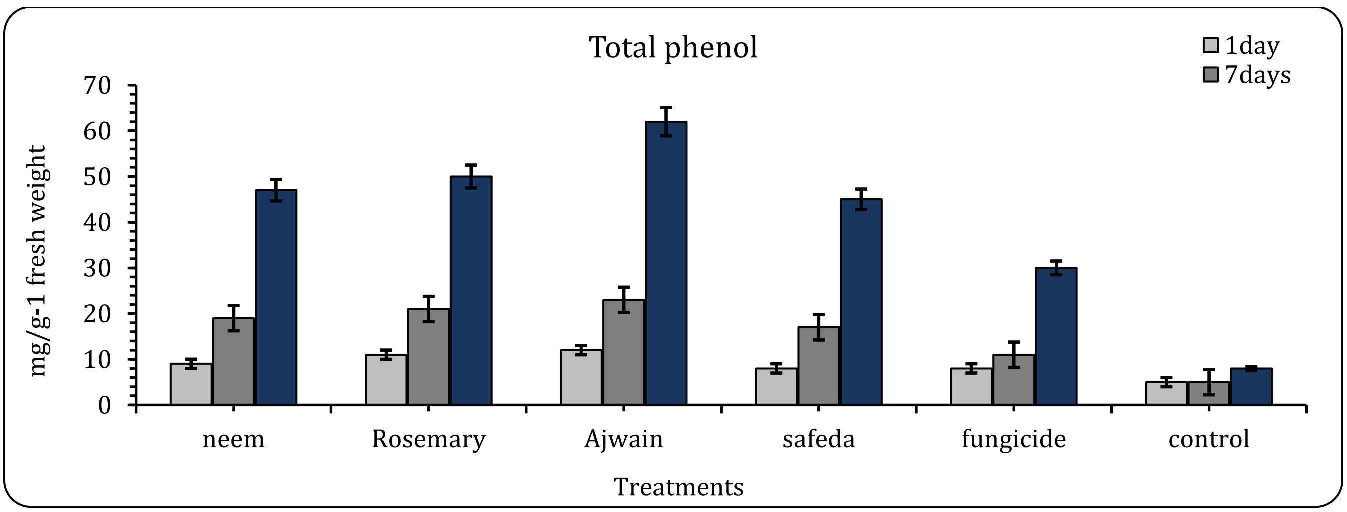

Figure 2. Comparison of total phenol contents of wheat leaves treated with different botanicals during infection of *Puccinia triticina* Eriks.

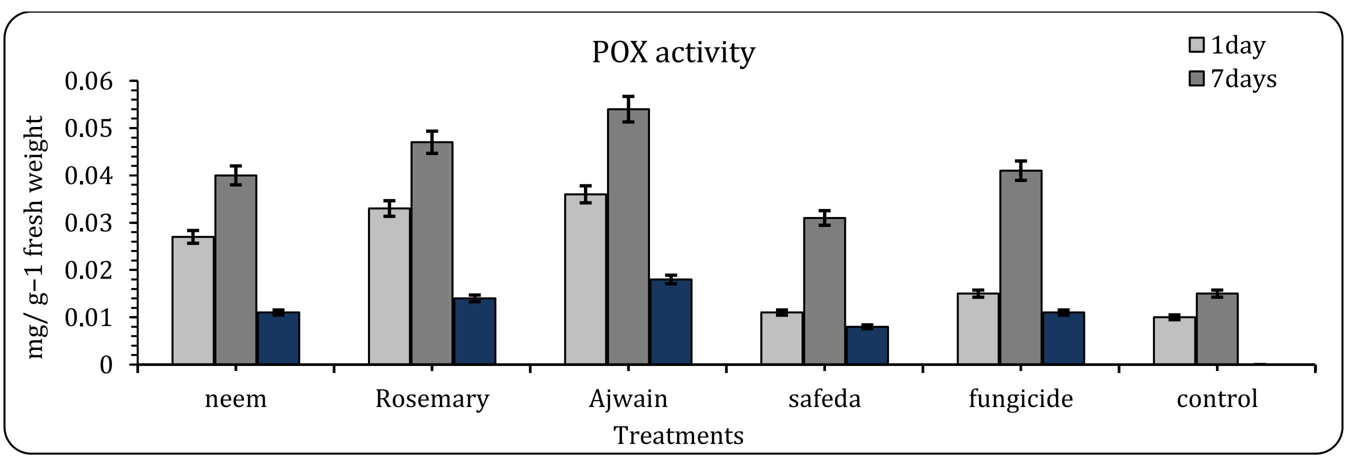

Figure 3. Comparison of peroxidase enzyme activity (POX) in wheat leaves treated with different botanicals during infection of *Puccinia triticina* Eriks.

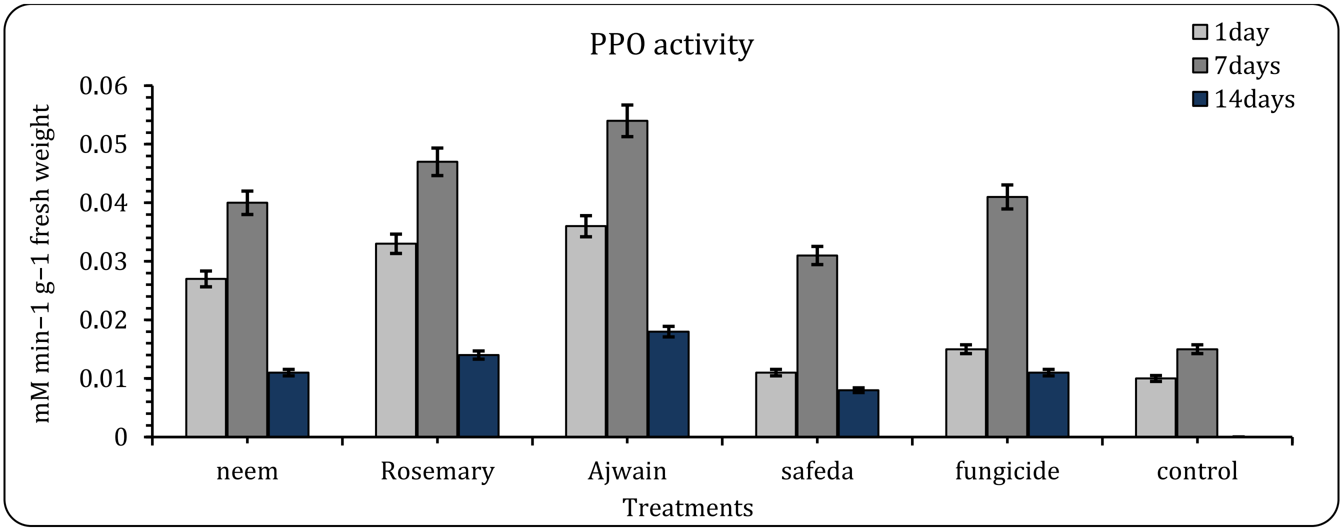

Figure 4. Comparison of polyphenol oxidase enzyme activity (PPO) in wheat leaves treated with different botanicals during infection of *Puccinia triticina* Eriks.

DISCUSSION

Finding of present experiment showed that all the plant extracts including rosemary, safeda, neem and ajwain were excellent defense inducers against leaf rust of wheat under natural conditions. Many past experiments were conducted on plant extracts to evaluate their effect on different pathogens, and some are much similar to our study. During an experiment Shabana *et al.* (2017) reported that incidence of leaf rust in wheat is significantly controlled by using different plant extracts including (clove, garlic, Brazilian pepper, white cedar, neem etc.) by spraying before the onset of disease. In another experiment methanol extracts of *curcuma zedoaria* was used against leaf rust of wheat and it exhibited strong activity against leaf rust of wheat by reducing (43, 80 and 100%) severity of disease (Han *et al.,* 2017). Foliar spray of various extracts as inducers before inoculation of *Alternaria solani* on potato plants led to decreases the severity of disease on potato plants. Foliar spray of neem, eucalyptus, pomegranate and garlic significantly decreases the severity of leaf rust of wheat (Kumar *et al.,* 2017; Nagwa and Abbas, 2017). Instable constituents extracted from *Lantana camara* leaf, flowers and stem showed maximum inhibitory effect against various pathogens including; *Pythium ultimum*, *R. solani*, *F. oxysporum* and *verticillium dahlia* (Boughalleb *et al.,* 2005). Result of our experiment showed that yield components of infected plants of wheat can be increased by using plant extracts. Findings of present experiment are in line with the work conducted by Shabana *et al.* (2017) who stated that yield component (Thousand-kernel weight) improved by (13.02, 13.81 and 15.73%) in infected wheat plant by using garlic, cedar and Brazilian pepper as compared to untreated control treatment. Abe Malik and Abbas, (2017) also described similar results.

Ajwain extract was found to be most effective in increasing the chlorophyll contents of infected leaves of wheat after first, seventh and fourteenth days of spray with (3.3, 3.8 and 4.2 mg/g), followed by rosemary (3.2, 3.5 and 3.9 mg chlorophyll/g fresh weight) respectively as compared to control (2.5, 2.3 and 1.9 mg/g) and fungicide treatment (2.6, 3 and 3.6 mg/g). Induced resistance is an important and environmentally friendly tool to prevent the crop from devastating broad-spectrum pathogens (Durrant and Dong, 2004). Through an induced resistant, synthesis of certain types of chemical compounds increase that can inhibit pathogen growth and invasion (Agrios, 2005). It was estimated that all botanical extracts significantly increase the phenol contents of infected plants as compared to non-treated control. Phenol contents gradually increase after spraying with plant extracts. Secondary metabolites viz; phenols, quinones, phenolic acid, flavonoids and cumarins were produced by all plants because they have ability to produce these kind of components (Cowan, 1999). It was noticed in many studies that components which contains phenolic structures viz; thymol, carvacrol and eugenol are more effective against plant pathogens. Results of the present study are similar with Karavaev *et* *al.* (2002) results, according to experiment results various botanical extracts of (Populous tremula (aspen), padus avium (bird cherry) and Chelidonium majus (swallow wort) plants effectively suppress the incidence of *P. triticina* because high phenolic compounds were recorded in the leaves of all plants.

Increased activity of enzymes (POX) peroxidase, (PPO) polyphenol oxidase was observed in all plants treated with plant extracts. In the past studies no mechanism of disease dominance by botanical extracts clearly understood, but there are much evidence in which involvement of botanical extracts as an inducer were deliberated (Fokkema, 1993). In the present experiment substantial increase in enzymes activity was observed in the plants treated with botanical extracts supported by the work of Kumar *et al.* (2017) and Karavaev *et al.* (2002). In both experiments aqueous extracts of different plant were tested against P. triticina and it was noticed that all plant extracts of (Populous tremula, padus avium and Chelidoniummajus) increases PPO activity in infected leaves of wheat which suppress the disease incidence of leaf rust. An increased PPO activity was observed in potato leaves when treated with botanical extract of *L. camara* (Kumar *et al.,* 2010). Many abiotic compounds including botanical extracts were used to induce resistance in plants against pathogens and the whole phenomenon of inducing resistance considered environmentally friendly (Morsy *et al.,* 2011). In another study, Swamy *et al.* (2015) stated that 32 bioactive components were found in *L. camara*. Antioxidant activity of *A. indica* was tested and results of experiment demonstrated that it has high antioxidant activity on phytochemicals (Satya Prasad *et al.,* 2015). Leaf extracts of *A. wilkesiana* was analyzed to check the rate of various compounds and result of the experiment demonstrated that all leaves contain high number of tannins and glycoside, and low number of alkaloids and cardiac flavonoids were also present. All the compounds play a vital role in the defense mechanism against lethal pathogens especially in case of leaf rust (Awe *et al.,* 2013). Botanical extracts are cost-effective, eco-friendly and can be prepared easily so they provide a better and cost-effective way to control the pathogens.

CONCLUSION

From the present study it is concluded that botanical extracts (Ajwain, Rosemary, Neem and Safeda) prove to be most conducive as an inducer against leaf rust caused by *P. triticina*. It is estimated through biochemical changes that, all tested botanical decreases the severity of leaf rust of wheat resulting in increasing chlorophyll and phenol contents. Oxidative activity of enzymes including POX and PPO also increases in those plants which are treated with botanical extracts. These enzymes are responsible for protecting wheat plants against rust pathogen.

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