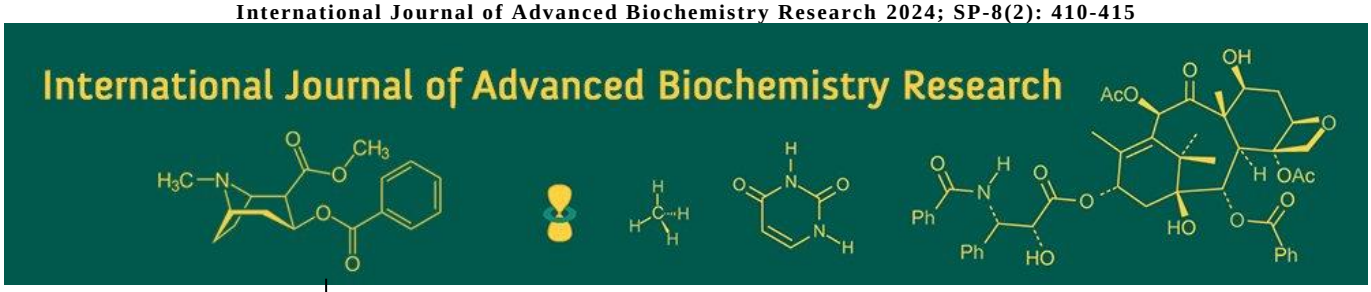

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Physiological and biochemical changes in contrasting chickpea genotypes under Fusarium wilt stress

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Abstract
Chickpea is an important pulse crop grown worldwide for its rich protein content. However, the yield of the crop is adversely affected by various biotic and abiotic stresses. Among biotic stress, Fusarium wilt (FW) caused by *Fusarium oxysporum* f.sp. *ciceris* (Foc) adversely affect the chickpea production and can cause 100% yield loss. The plant also possesses defense mechanism to counteract the pathogen attack by employing various morphological, physiological, biochemical and molecular changes during this host-pathogen interaction. The present study aims to understand the physiological and biochemical changes that occur during chickpea-Foc interaction in two contrasting genotypes, FW-resistant (Pusa Green 112) and FW-susceptible (ILC 482). Drastic changes in physiological parameters like relative water content (RWC) and electrolyte leakage (EL) were observed for chickpea genotypes under Fusarium infection. The RWC was heavily compromised in susceptible genotype (ILC 482) and electrolyte leakage was elevated in this genotype under Foc stress. While, no change in RWC and EL was observed for resistant genotype (Pusa Green 112) suggesting a robust resistance mechanism operating in this genotype. Alteration in antioxidant activities were also observed in the genotypes under Foc attack. SOD activity was higher in Pusa Green 112 under Foc stress suggesting an efficient ROS scavenging system present in this genotype. Exaggerated CAT activity in ILC 482 (susceptible) suggests excessive ROS production. Such drastic increase in CAT activity at symptom development stage is a late response to impart protection against pathogen infection. Furthermore, the MDA level was also compromised in ILC 482, suggesting heavy membrane damage due to Foc attack. Hence, the present study provides new insights into physiological and biochemical changes that occurs during chickpea-Foc interaction.

Keywords: Chickpea, Fusarium wilt, Relative water content, Antioxidant enzymes, malondialdehyde

Introduction
Chickpea (*Cicer arietinum* L.) is a legume crop grown for its good dietary protein content. It is the topmost pulse crop in India and grown in a land area of 10.94 Mha with an annual production of 11.9 Mt (FAOSTAT, 2021) [1]. At global level, chickpea is the 3rd topmost pulse crop and grown in an area of 15 Mha with an annual production of 15.88Mt (FAOSTAT, 2021) [1]. The average productivity of chickpea is very low (1.05 t/ha) as compared to its genetic potential of 6 t/ha (Parween *et al*., 2015; FAOSTAT, 2021) [2, 1]. This huge difference in productivity is mainly attributed to various biotic stresses like Ascochyta blight, Fusarium wilt, pod borer infestation and abiotic stresses like drought, salinity, heat and cold (Kashiwagi *et al*., 2015) [3]. Among biotic stresses, Fusarium wilt is one of the most devastating fungal diseases in chickpea and accounts for 100% yield loss (Yadav *et al*., 2023) [4]. Fusarium wilt is caused by a soil-borne and hemibiotrophic fungus, *Fusarium oxysporum* f. sp. *ciceris* (Foc). The pathogen has the characteristics features of both biotrophs and necrotrophs (Lyons, *et al*., 2015) [5]. The infection cycle starts with biotrophic phase and then switch to necrotrophic phase at later stage of infection. The fungus spreads through infested soil and contaminated crop residues. The pathogen enters the host through root tips and mainly clogs the xylem vessels via mycelia. Clogging of xylem leads to blockage of water transport preventing movement of water to the upper parts of the plant. This scarcity in water leads to wilting followed by complete death of plant (Gupta *et al*., 2013) [6]. To counteract the pathogen attack, the plant employs a two-layered innate immunity system, pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). This immunity is achieved by employing various physiological, biochemical and molecular changes during chickpea-Foc interaction.

Physiological changes include reduced relative water content, electrolyte leakage, changes in chlorophyll fluorescence activity as well as pigment content (Bhar *et al*., 2017) [7]. Biochemical changes include ROS burst, higher ROS scavenging activity, lipid peroxidation, production of phytohormones like SA, JA (Garcia-Limones *et al*., 2002; Gupta *et al*., 2013; Bhar *et al*., 2017; 2018) [8, 7, 6]. The molecular changes include induction of various defense signaling molecules, defense genes like PR genes, various transcription factors (Chakraborty *et al*., 2020).

The present study is done to understand the physiological and biochemical alterations in chickpea under Fusarium wilt stress. Two chickpea genotypes contrasting for Fusarium wilt resistance, ILC 482 (FW-susceptible) and Pusa Green 112 (FW-resistant), were studied for various physiological and biochemical parameters at two time points of infection, 2- and 10-days post inoculation (DPI).

Materials and Methods

Plant infection assay: The chickpea seeds were surface sterilized using 0.1% mercuric chloride for 5 min followed by five washes with double distilled water (ddH_2O) for 5 min each. The seeds were sown in medium-sized clean plastic pots (3 seeds/pot) filled with autoclaved soil rite-mix. The pots were then transferred to a growth chamber with the following conditions- 14 h light/10 h dark, temperature range 22-25 °C and relative humidity (RH) 60 –70%, and light intensity of 135 µmol m^{-2} s^{-1}. The plant infection was done by root clipping method as described by Tullu *et al*. (1998) [8]. For stress samples, the twelve days old seedlings were infected with Foc spores (10^6/ml). For control samples, the seedlings were treated with ddH_2O. The control samples were designated as PC (Pusa Green 112 Control), IC (ILC 482 Control), and stress samples were designated as PS (Pusa Green 112 Stress), IS (ILC 482 Stress).

Physiological parameters

Estimation of Relative Water Content (RWC)

The protocol for RWC was adopted by Bhar *et al*. (2018) [9] with slight modifications. The fully expanded leaf from the top of the plant was taken for the study. The RWC was calculated using the formula given below.

RWC (%) = [(Fresh weight - Dry weight) / (Turgid Weight - Dry Weight)] x 100.

Measurement of electrolyte leakage (EL)

Electrolyte leakage (EL) was measured according to the protocol given by Jatan *et al*. (2019) [11]. The electrolyte leakage was measured based on the formula given below.

$$EL\ (\%) = \frac{E1}{E2} \times 100$$

Antioxidant activity assay

GPX activity assay

The GPX activity was estimated using the protocol given by Hameed *et al*. (2014) [12]. The activity was estimated by using pooled samples of shoot and root tissues with 100 mg each. The enzymatic reaction was initiated and increase in absorbance of the reaction was measured at 470 nm. Absorbance was recorded after every 20 s for 1 min. The enzyme activity was calculated based on tissue fresh weight (U/g FW).

CAT activity assay

The CAT activity was estimated using the protocol given by Chance and Maehly (1955) [13]. The activity was estimated by using pooled samples of shoot and root tissues with 100 mg each. The enzymatic reaction was initiated and immediately the decrease in absorbance of the reaction was measured at 240 nm. Absorbance was recorded after every 20 s for 1 min in a UV-Vis spectrometer (Lambda 35, Perkin Elmer Inc., USA). The enzyme activity was calculated on the basis of tissue fresh weight (U/g FW).

APX activity assay

The APX activity was estimated using the protocol given by Chen and Asada (1989) [14]. The activity was estimated by using pooled samples of shoot and root tissues with 100 mg each. The enzymatic reaction was initiated and immediately the decrease in absorbance of the reaction was measured at 290 nm. Absorbance was recorded after every 20 s for 1 min. The enzyme activity was calculated based on tissue fresh weight (U/g FW).

SOD activity assay

The SOD activity was estimated using the protocol given by Dhindsa *et al*. (1981) [15]. The activity was estimated by using pooled samples of shoot and root tissues with 100 mg each. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium (NBT) photoreduction rate. The enzyme activity was calculated based on tissue fresh weight (U/g FW).

Estimation of Malondialdehyde (MDA)

The MDA was estimated to check the degree of lipid peroxidation by using the protocol given by Viswakarma *et al*. (2015). The MDA level was estimated by using pooled samples of shoot and root tissues with 100 mg each. The absorbance was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm, The MDA content was calculated based on fresh weight (nmol/g FW).

The formula for the calculation of MDA content is given below

$$MDA\ (nmol/g\ FW) = \frac{(A532 - A600) \times V \times 1000}{\varepsilon \times FW}$$

Where, ε = Extinction coefficient of MDA-TBA abduct at 532 nm = 15 mM^{-1} cm^{-1}

V = Volume of crude extract

Statistical Analysis

The physiological and biochemical data were analyzed by using one-way analysis of variance (ANOVA) with post-hoc Tukey HSD (Honestly significant difference) (https://www.socscistatistics.com/) to test statistical significance between means of control and stress treatments at *P*-value < 0.05. The figure's values represent mean ± SE (n = 3) of three biological replicates per genotype per treatment.

Results

The Fusarium wilt symptoms like drooping of leaflets and stunted growth were observed at 12 days post inoculation (dpi) in susceptible genotype, ILC 482 while no such symptoms were observed for resistant genotype, Pusa Green 112. Based on the phenotypic changes, we carried out the physiological and biochemical studies of control and stress samples at 12 dpi.

Physiological changes in contrasting chickpea genotypes under Fusarium wilt stress: RWC was studied to understand the effect of Fusarium infection on water status in plants, as FW affects the water transport by clogging the xylem vessels by mycelia deposition. A drastic reduction in RWC was observed for ILC 482 (IS-12 dpi- 59.5%) under Foc stress at late time point (12 dpi) as compared to its control counterpart (IC-12 dpi- 76.5%) as well as stressed Pusa Green 112 (PS-12 dpi-76.3%) ($p< 0.05$) (Fig. 1a).

EL assay was done to understand the degree of membrane damage occurring due to Fusarium infection. An increase in the electrolyte leakage was observed for ILC 482 (34%) under Foc stress ($p< 0.05$). On the other hand, the resistant genotype, Pusa Green 112 (24.5%) did not show any remarkable change in electrolyte leakage status after Foc stress at this time point (Fig. 1b).

Biochemical changes in contrasting chickpea genotypes under Fusarium wilt stress: The activity of four major antioxidant enzymes, namely, GPX, CAT, APX, and SOD were measured to determine their role in chickpea defense against wilt stress. GPX activity assay revealed that both ILC 482 (79.3 U/g FW) and Pusa Green 112 had similar level of GPX activity (89.1 U/g FW) under Foc stress ($p< 0.05$) (Fig. 2a).

CAT activity assay revealed that the activity is tremendously higher in ILC 482 (133 U/g FW) as compared to Pusa Green 112 (89.4) under Foc stress (Fig. 2b).

APX activity assay revealed similar level of APX activities in both ILC 482 (3.7 U/ g FW) and Pusa Green 112 (3.4 U/ g FW) under Foc stress (Fig. 2c).

SOD activity assay showed slightly lower level of SOD activity in ILC 482 (132 U/ g FW) as compared to Pusa Green 112 (143 U/ g FW) ($p< 0.05$) (Fig. 2d).

The rate of lipid peroxidation in chickpea genotypes under control and stress conditions were measured by quantifying the MDA levels, a by-product of lipid peroxidation process. The susceptible genotype, ILC 482 (7.9 nmol/g FW) showed significantly higher MDA levels as compared to the resistant genotype, Pusa green 112 (PS-10 dpi-6.2 nmol/g FW) under Foc stress. The higher level of MDA in susceptible genotype under Foc stress hints that membrane damage is caused due to Foc attack (Fig. 3).

Discussion

Relative water content (RWC) is a useful indicator of water status in plants (Soltys-Kalina *et al*., 2016) [16]. The exposure of Fusarium infection led to reduced relative water content in susceptible genotypes ILC 482 as compared to resistant genotype, Pusa Green 112 at 12 dpi. This suggests wilting occurs due to severe water shortage caused by clogging of xylem by fungal mycelia in the ILC 482 roots. Electrolyte leakage indicates the degree of cell membrane instability which in turn gives an idea about the cell death (Hatsugai and Katagiri, 2018) [17]. The electrolyte leakage was severely high in susceptible genotype as compared to resistant one suggesting occurrence of severe membrane damage and cell death due to Fusarium infection.

Production of ROS (Reactive Oxygen Species) like hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) (oxidative burst) is one of the earliest defense responses against pathogen attack (Vlot *et al*., 2009).

However, excess ROS can damage cellular components due to their toxic effect. Production of antioxidants are indicative of resistance against Fusarium attack as these enzymes remove the excess ROS produced during oxidative burst (Narula *et al*., 2020) [18]. Antioxidants (also known as defense enzymes, ROS scavengers) such as GPX, CAT, APX detoxify the most abundant ROS, H_2O_2, and SOD detoxify the superoxide radicals thereby maintaining a ROS homeostasis inside the plant cell (Wojtaszek, 1997) [19]. In ILC 482 (susceptible), the enzyme activities were similar (in case of GPX, APX), higher (in case of CAT) and slightly reduced (in case of SOD) as compared to Pusa Green 112 (resistant) at 12 dpi. Exaggerated CAT activity in ILC 482 (susceptible) suggests excessive ROS production. Similar observation was also observed for another study on chickpea infected with Foc Race 5, where susceptible genotype, JG 62, showed significantly higher CAT activity (Garcia Limones *et al*., 2002) [8].

Such drastic increase in CAT activity at symptom development stage is a late response to impart protection against pathogen infection. Furthermore, lower SOD activity under Foc infection in susceptible genotype, JG 62 confirm inefficient antioxidant system during the infection process. While the resistant genotype, Pusa Green 112 showed higher SOD activity at this time point suggesting robust ROS scavenging mechanism that imparts resistance against the pathogen. MDA (Malondialdehyde) is the final product of the peroxidation of unsaturated fatty acids in phospholipids present in the plant membrane and signifies the degrees of membrane damage (Ayala *et al*., 2014) [20]. A significantly higher peak in MDA level in wilt infected susceptible genotype as compared to resistant genotype at both the time points means that the membrane lipids have been heavily damaged in susceptible genotype due to Foc invasion.

Hence, the present study provides new insights into physiological and biochemical changes that occurs during chickpea-Foc interaction. Further studies on molecular mechanism involved during this interaction will give a clearer picture regarding the chickpea defense against Foc infection.

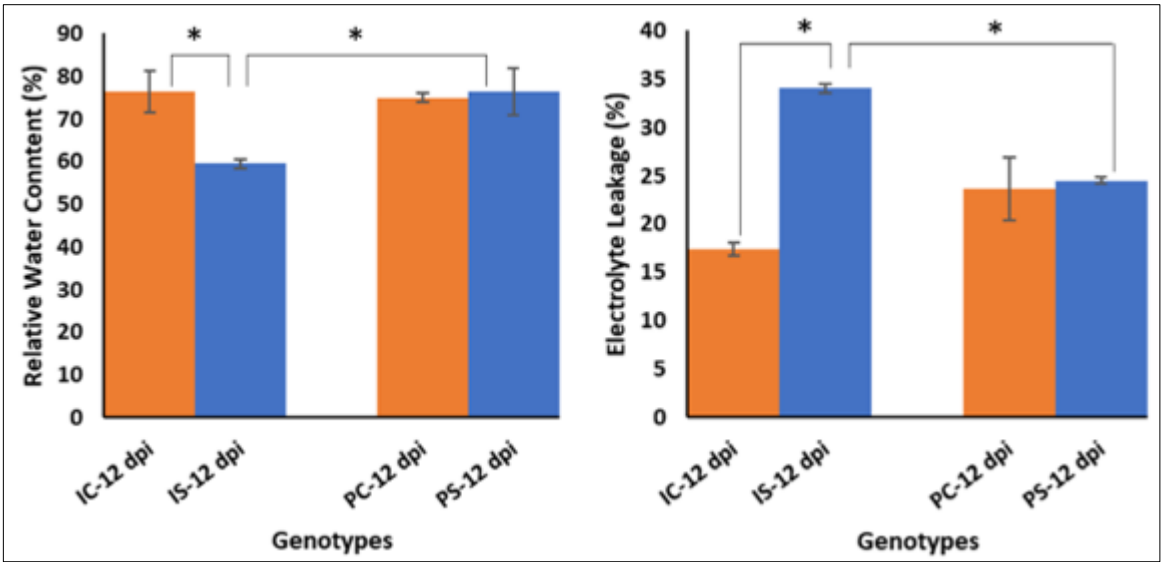

Fig 1: Physiological changes in chickpea genotypes in response to Foc stress.

(a) Relative water content (RWC) and (b) Electrolyte leakage (EL) were calculated for ILC 482 (FW-susceptible), Pusa Green 112 (FW-resistant), RWC and EL were calculated under control and Foc stress conditions at 12 dpi. The values are expressed in percentage (%). The values are means of three biological replicates and vertical bar represents standard errors of means of three biological replicates. * Represent significant differences between the treatments at $p< 0.05$.

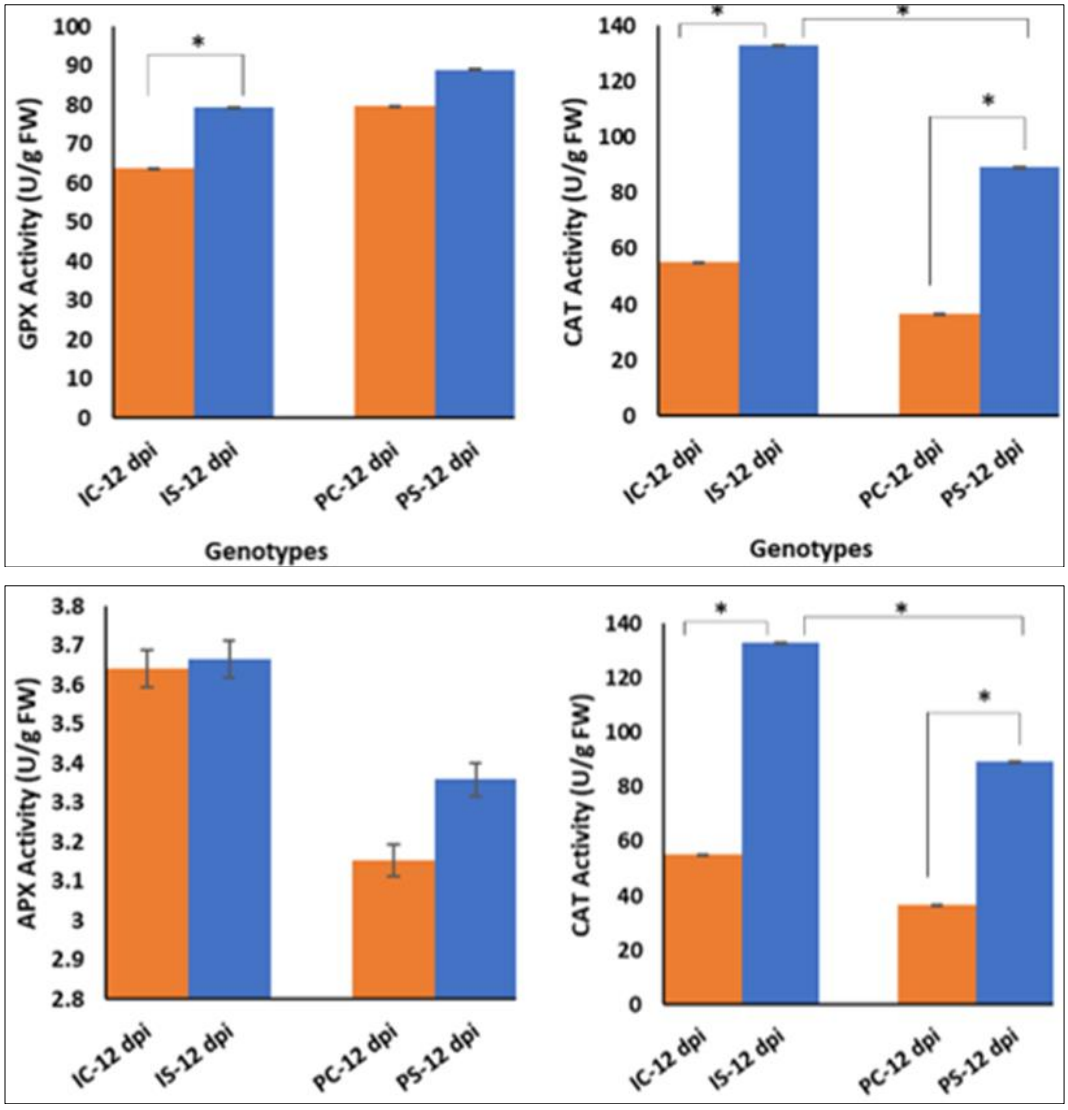

Fig 2: Changes in antioxidant enzymes in chickpea genotypes in response to Foc stress

The antioxidant enzymes assay of four defensive enzymes were carried out by spectrophotometric method. This include (a) GPX activity (b) CAT activity (c) APX activity and (d) SOD activity of in ILC 482 (FW-susceptible) and Pusa Green 112 (FW-resistant) under control and Foc stress conditions at 12 dpi. The values are means of three biological replicates and vertical bar represents standard errors of means of three biological replicates. * Represent significant differences between the treatments at $p< 0.05$.

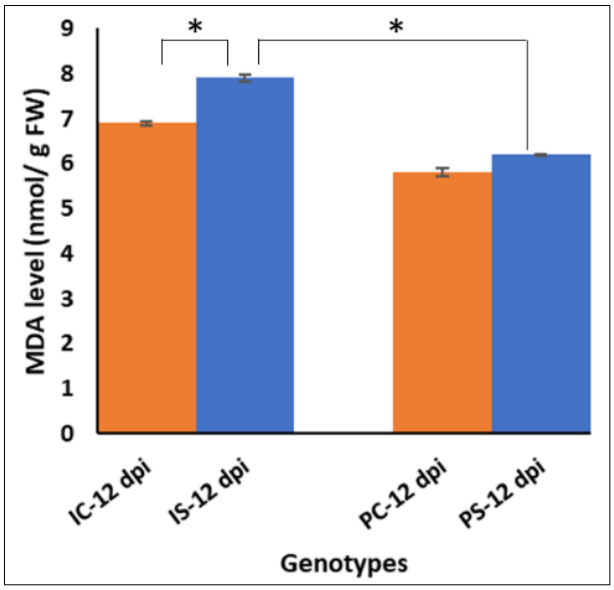

Fig 3: Changes in MDA level in chickpea genotypes under Foc stress.

Level of MDA was estimated to determine the degree of membrane damage in ILC 482 (FW-susceptible) and Pusa Green 112 (FW-resistant) under control and Foc stress conditions at 12 dpi. The values are means of three biological replicates and vertical bar represents standard errors of means of three biological replicates. * Represent significant differences between the treatments at $p< 0.05$.

Conclusion

The study on Fusarium wilt (Foc) stress in chickpea genotypes revealed significant differences in physiological and biochemical responses between the susceptible genotype ILC 482 and the resistant genotype Pusa Green 112. The physiological assessments indicated a substantial reduction in relative water content (RWC) and an increase in electrolyte leakage (EL) in ILC 482 under Foc stress, highlighting the genotype's susceptibility to Fusarium infection through its impact on water transport and membrane integrity. Conversely, Pusa Green 112 demonstrated resilience, maintaining stable RWC and EL levels, indicative of its resistance to Foc stress.

Biochemically, the activities of antioxidant enzymes (GPX, CAT, APX, and SOD) and levels of lipid peroxidation (measured as MDA) were analysed to understand the oxidative stress response. Both genotypes exhibited similar GPX and APX activities, suggesting a basic level of defense against Foc-induced oxidative stress. However, significant differences were observed in CAT and SOD activities, with ILC 482 showing higher CAT activity but slightly lower SOD activity compared to Pusa Green 112. The higher MDA levels in ILC 482 further confirmed the susceptibility of this genotype to membrane damage under Foc stress.

These findings underscore the complexity of the plant defense mechanism against Fusarium wilt, involving both physiological alterations and oxidative stress management. The resistance observed in Pusa Green 112 is likely attributed to its efficient anti-oxidative defense system and better maintenance of cellular integrity under stress. Understanding these contrasting responses provides valuable insights into the mechanisms of resistance and susceptibility in chickpea genotypes, offering potential pathways for breeding Fusarium wilt-resistant chickpea varieties.

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