The Pharma Innovation Journal 2022; 11(2): 1897-1900

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(2): 1897-1900
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www.thepharmajournal.com
Received: 10-12-2021
Accepted: 08-01-2022

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Influence of *Colletotrichum lindemuthianum* infection on the biochemical defence response in leaves of common bean (*Phaseolus vulgaris* L.)

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Abstract
Common bean infected with *Colletotrichum lindemuthianum* negatively effects the growth and development of bean plants. It is hypothesised that pathogen-plant interactions usually induce hypersensitive response by generating H_20_2 and lipid peroxidation of cell membrane in response to fungal infections. This study was conducted in common bean inoculated with pathogen *C. lindemuthianum* of two susceptible (BR 104 & BR 50) and one resistant (VL 63) common bean varieties. The pathogen was inoculated using leaf spray method during early flowering stage and plants were observed for changes at 3rd DAI, 6th DAI and 9th DAI. The leaf samples were collected to know the biochemical defense responses generated after inoculation of the pathogen. On 3rd day, electrolyte leakage percentage was maximally increased in VL 63 and BR 104 inoculated with T1 followed by T2 as compared to control. In contrast, on 6th day highest percent increase was recorded with T1 treatment in BR 50 whereas least percent increase was recorded in VL 63 inoculated with T2 compared to control. In response to *C. lindemuthianum* infection in common bean, the H_2O_2 levels significantly increase in resistant variety VL 63 during 3rd and 6th days of inoculation however, the susceptible varieties of common bean displayed no significant changes. The level of lipid peroxidation was significantly enhanced on 6th and 9th day as compared with uninoculated control plant in BR50 These results suggest that bean infected with *C. lindemuthianum* displays various biochemical responses an infected with *C. lindemuthianum* as a defense mechanism with days of disease development.

Keywords: *Phaseolus vulgaris* L., electrolyte leakage, lipid peroxidation, H_2O_2

Introduction
Common bean (*Phaseolus vulgaris* L.) is a well-known domesticated legume crop grown widely all over the world providing an important economy for marginal farmers in the developing countries and a high value commodity crop with high nutritional quality in the developed nation (Schwartz, 2006) [19]. It is a staple pulse food in many areas of the tropics and sub-tropics and make significant contributions to food security, income, and soil fertility (Beebe, 2012) [4]. Anthracnose disease caused by the pathogen *Colletotrichum lindemuthianum*, is one of the most important seed borne disease in common bean all around the world. It is considered as one of the most economically vital fungal disease causing huge devastation to farmers' income, resulting in yield losses as high as 50% to 90% in severe cases (Ntui *et al*., 2021) [16]. The role of antioxidant enzymes, hydrogen peroxide and PR proteins in the compatible and incompatible interactions of Cowpea (*Vigna unguiculata*) genotypes with the fungus *Colletotrichum gloeosporioides* was well studied by Oliveira *et al.* (2014) [17]. Similarly, Eloy *et al.* (2015) [7] reported that H_2O_2 manipulation by pharmacological compounds could alter the lifestyle of *Colletotrichum gloeosporioides* during interaction with the Tracuateua cowpea genotype. Another study by Hasim, (2017) [11] measured the changes of hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and total ion leakage in the leaves of two coconut cultivars. Resistant cultivar showed higher amount of H_2O_2, lipid peroxidation and total ion leakage compared to susceptible cultivar especially at 21 days of experiment in common bean. Siddique *et al.* (2014) [20] found that the amount of MDA was significantly higher in leaves of inoculated plants of both resistant genotypes as in non-inoculated plants and decreased in inoculated plants of both susceptible genotypes over their healthy plants. The biochemical features associated with soybean plant resistance to target spot disease caused by fungus *Corynespora cassiicola* was determined by Fortunato *et al.* (2015) [8].

Material and Methods

The seeds of common beans varieties (BR 104 and BR 50) were procured from RARSS, Bhaderwah, SKUAST-J, Main Campus, Chatha and VL-63, an anthracnose resistant variety of common bean from ICAR-VPKAS, Almora, and Uttarakhand, India. Leaves and pods of common bean *(Phaseolus vulgaris)* displaying the symptoms of anthracnose caused by *Colletotrichum lindemuthianum* were collected from Bhaderwah region. The sample were properly wrapped in polythene bag and immediately brought to the laboratory of Division of Biochemistry, Faculty of Basic Sciences, SKUAST-Jammu, J&K and pathogen was isolated, purified and multiplied.

Foliar spray inoculation was done with modified methods of (Posada *et al*., 2007) [18]. The foliar spray inoculation method was performed with a hand sprayer and an average of 5 ml per plant was applied. The spray was directed to the lower and upper surface of leaves. Controls sprayed with water in the same way as each treatment mentioned above. After spraying, each plant was covered with a plastic bag for 24 h to maintain a high level of humidity.

Hypersensitivity response was determined by ion leakage method (Hasim and Yusuf 2017) [11]. 200 mg leaf sample was rinsed with deionized water and placed in a test tube containing 10 ml of deionized water at 40°C for 30 minutes. Then, using the electric conductivity meter (EC meter), the conductance (C1) was measured. The percentage of leakage was calculated according to the formula below:

Ion leakage % = C1/C2 x 100

Hydrogen peroxide levels were measured calorimetrically as described by Velikova *et al*. 2000 [23] with slight modification. Fresh leaf sample (150mg) was homogenized on ice-bath in 1ml of 0.1% Trichloroacetic acid (TCA) and centrifuged at 10,000 rpm (Eppendorf, Pvt Ltd, Germany) for 10 minutes at 4°C. The reaction was started by the addition of 1.0 mL of 1.0 M potassium iodide (KI, freshly prepared). Reaction mixture without KI was used as a blank. The H_2O_2 concentration was calculated based on the standard curve prepared using 0.1 to 1mM H_2O_2.

Lipid peroxidation (LPO) was referred to malondialdehyde (MDA) contents estimated as Thiobarbituric acid (TBA) (Heath and Packer 1968) with slight modifications. The absorbance of the reaction was recorded at 532 nm with the value of non-specific absorption at 600 nm subtracted from the absorbance values. The MDA concentration was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as mole of MDA per g of fresh weight (FW) of sample as per the formula below:

$A=\epsilon bI$

Where
A=changes in absorbance,
l=cuvette length and
∈=extinction coefficient.

Result

The level of hypersensitivity response was measured in terms of electrolyte percentage. Compared with control, inoculation with *Colletotrichum lindemuthianum* significantly enhanced on 3rd, 6th and 9th days after inoculation in VL 63 and BR 104 whereas in BR 50 on 6th and 9th day as shown in Table 1. On 3rd day, electrolyte percentage was maximally increased in VL 63 and BR 104 inoculated with T1 followed by T2 as compared to control. In contrast on 6thday highest percent increase of 87.48% was recorded with T1 treatment in BR 50 whereas least percent increase of 53.45% was recorded in VL 63 inoculated with T2 compared to control. On 9th day, maximum percent increase was recorded in BR50 inoculated with T2 followed by T1 while lowest increase of 18.34% (VL63) was recorded when inoculated with T2 relative to uninoculated plant.

In response to *C. lindemuthianum* infection in common bean, the H_2O_2 levels significantly increase in resistant variety VL 63 after 3rd and 6th days of inoculation shown in Table 2. In contrast both the susceptible varieties of common bean displayed no significant changes. On 3rd day, the levels of H_2O_2 was found to be highest increased in VL 63 while least increase was recorded in BR 50. Similarly, on 6th day, the accumulation of H_2O_2 showed maximum in VL 63 with T1 and T2 treatments as compared to uninoculated control. However, on 9thday, highest H_2O_2 production highest increase when inoculated T1 followed T2 in BR 104 and while least percent increase of 21.45% (VL 63) was observed in beans inoculated with T2 as compared to control.

The level of lipid peroxidation was measured in terms of MDA content, a product of lipid peroxidation when compared with control, inoculation with *Colletotrichum lindemuthianum* insignificantly enhanced lipid peroxidation in VL 63 and BR 104 whereas in BR50 it was found to be significantly enhanced on 6th and 9th day, accumulation MDA content shown in Table 3. On 3rd day, MDA content was maximally increase in VL 63 and BR 104 inoculated with T1 followed by T2 respectively as compared to control. In contrast, on 6th day highest percent increase was recorded in BR 50 inoculated with T1 whereas least percent increase was recorded in VL 63 beans inoculated with T2 as compared to control. On 9th day, maximum percent increase of 3974% (BR 50) followed by 33.33% (BR 104) inoculated with T1 while lowest increase was found in VL 63 when inoculated with T2 relative to uninoculated control plant.

Table 1: Hypersensitive response (% total ion leakage) in leaves of resistant and susceptible varieties of common bean (*Phaseolus vulgaris* L) at different days after inoculation (DAI) with *C. lindemuthianum*

Days / Isolates	BR-104			Mean	BR-50			Mean	VL-63			Mean
	Days				Days				Days			
	3rd	6th	9th		3rd	6th	9th		3rd	6th	9th	
Control	25.53^{a}	27.48^{c}	26.55^{e}	26.52	23.09^{a}	24.96^{b}	23.78^{d}	23.94	21.71^{a}	22.00^{c}	22.00^{e}	21.90
Bhaderwah	28.49^{b}	50.53^{d}	48.04^{f}	42.35	24.96^{a}	46.79^{c}	43.19^{e}	38.31	35.42^{b}	34.42^{d}	26.42^{f}	32.09
IARI	28.23^{b}	49.84^{d}	47.73^{f}	41.93	24.70^{a}	46.08^{c}	43.47^{e}	38.08	33.87^{b}	33.76^{d}	26.04^{f}	31.22
Mean	27.42	42.62	40.77		24.25	39.28	36.81		30.33	30.06	24.82	
CD $_{(p\leq 0.01)}$ (I × D)	2.48				2.74				2.05			

Table 2: Hydrogen peroxide (H_2O_2) concentration (µM/g FW) in inoculated and control leaves of resistant and susceptible varieties of common bean (*Phaseolus vulgaris* L) at different days after inoculation (DAI) with *C. lindemuthianum*

Days / Isolates	BR-104			Mean	BR-50			Mean	VL-63			Mean
	Days				Days				Days			
	3rd	6th	9th		3rd	6th	9th		3rd	6th	9th	
Control	6.21a	6.33b	6.29c	6.28	7.88a	7.87b	7.88c	7.87	4.98a	5.03c	5.10e	5.03
Bhaderwah	7.71a	8.47b	8.02c	8.07	9.55a	10.21b	9.75c	9.83	9.72b	7.72d	6.34e	7.93
IARI	7.67a	8.38b	7.96c	8.00	9.20a	10.13b	9.62c	9.65	9.47b	7.56d	6.19e	7.74
Mean	7.20	7.73	7.42		8.87	9.40	9.08		8.05a	6.77	5.87	
CD $_{(p\leq 0.01)}$ (I × D)	2.17				2.56				2.43			

Table 3: Leaf malondialdehyde concentration (µM/g FW) in inoculated and control leaves of resistant and susceptible varieties of common bean (*Phaseolus vulgaris* L) at different days after inoculation (DAI) with *C. lindemuthianum*

Days / Isolates	BR-104			Mean	BR-50			Mean	VL-63			Mean
	Days				Days				Days			
	3rd	6th	9th		3rd	6th	9th		3rd	6th	9th	
Control	6.74a	6.89b	6.95c	6.86	7.00a	7.12b	7.26d	7.13	5.80a	5.91b	6.10c	5.94
Bhaderwah	7.71a	9.25b	9.26c	8.74	7.68a	9.98c	10.15e	9.27	7.52a	7.14b	7.08c	7.24
IARI	7.66a	9.10b	9.08c	8.61	7.57a	9.62c	9.60e	8.93	7.32a	6.86b	6.92c	7.03
Mean	7.37	8.41	8.43		7.41	8.91	9.00		6.88	6.64	6.70	
CD $_{(p\leq 0.01)}$ (I × D)	2.43				2.13				1.90			

Discussion and Conclusion

Cellular membrane defect (increased permeability and ion leakage) caused by pathogen attack is one of the most common outcomes of infection and can be detected by electrolyte efflux. Electrolyte leakage values in common bean leaves were considerably elevated in both resistance and susceptible varieties in response to *C. lindemuthianum* infection in the current investigation, whereas the EL on the 6th and 9th DAI was higher in both susceptible varieties. The current findings are consistent with the findings of Chen *et al*. (2015) [5] who found that stripe rust infection increased electrolyte leakage in wheat susceptible cultivars (Sy95-71) more than resistant cultivars (CN19). According to Ketta (2015) [13], *F. virguliforme* infection increased electrolyte leakage in sensitive soybean plants compared to uninfected plants. Our findings revealed that pathogen-infected common bean plants lost electrolyte components as a result of pathogen infection.

Hydrogen peroxide (H_2O_2) is a versatile molecule that may be involved antimicrobial effects and is involved in other defense responses such as cell wall modification, lipid peroxidation, phytoalexin production, hypersensitive reaction (HR), and defense-related gene activation, as well as acting as an intra or intercellular signalling molecule in the activation of plant defence mechanisms against pathogen attack (Heller and Tudzynski, 2011) [12]. H_2O_2 aids in stress adaptation by providing tolerance at low concentrations, while at high concentrations it causes cellular damage and cell death (Vandenabeele *et al*. 2003; Stone and Yang 2006) [22, 21].

In the current study, considerable H_2O_2 accumulation was seen in the VL 63 variety on the 3rd and 6th DAI, whereas H_2O_2 build up was non-significant in the BR 104 and BR 50 varieties after inoculation with *C. lindemuthianum*. However, we confirmed that the protective antioxidant system was activated in the resistant common bean cultivar, resulting in a change in H_2O_2 levels and the avoidance of severe oxidative damage caused by H_2O_2 overproduction. Abdelaal *et al*. (2014) [1] made similar observations where that levels of ROS, primarily hydrogen peroxide (H_2O_2), increased significantly in resistant cultivars inoculated with Stripe rust fungus *P. striiformis.* According to Ge *et al.* (2013) [10], hydrogen peroxide build up was greater in leaves of resistant melon (*Cucumis melo* L.) cultivars than in susceptible cultivars infected with *Colletotrichum lagenarium.* Barreto *et al.* (2007) [3] found that the *C. gloeosporioides* resistant cowpea genotype TE97 accumulates more H_2O_2 than the susceptible BR3 genotype, which also accumulates H_2O_2 but to a lower level after *C. gloeosporioides* inoculation. According to our findings, the job of hydrogen peroxide (ROS) may be to kill or suppress pathogens by making local necrosis and by breaking off the penetrating hyphae of the fungus.

Malondialdehyde (MDA) formed during lipid peroxidation is a sign of pathogenic infection induced cellular damage at the cell membrane (Dallagnol *et al*. 2011; Aly *et al*. 2012) [6, 2]. An increase in MDA levels suggests the development of membrane damage caused by polyunsaturated fatty acid peroxidation, which results in the formation of ROS and subsequent oxidative stress (Montillet *et al.* 2005) [15]. The end product of lipid peroxidation may act as a signal to activate defense reactions (Kuzniak and Urbanek 2000) [14].

The current study findings revealed that *C. lindemuthianum* treatments resulted in a considerable rise in MDA content in both susceptible and resistant varieties. Variety VL 63 exhibited stronger defense in response to infection, with greater MDA at the early infection stage and later reduction, indicating anthracnose resistance potential. Our outcomes are consistent with Garcia-Limones *et al.* (2002) [9] who found that when chickpeas were infected with *Fusarium oxysporum*, the MDA level increased in both resistant and susceptible cultivars. This rise, however, was greater and happened earlier in the resistant cultivar. Chakraborty *et al*. (2019) found that as the infection (*Colletotrichum gloeosporioides*) progressed in common bean leaves, there was a gradual increase in MDA concentration, which remained significantly increased until the 4th DAI. All secondary metabolite analyzed here were found to be changing in root of inoculated plants compared to uninoculated plants in both resistant and susceptible varieties. This is an indication of systemic defense response in these varieties against the pathogen. Giving more attention to antioxidant enzymes activity and ROS levels are needed and might be useful for discovering a new alternative disease control strategy. This could decrease the

environmental pollutions as a result of using fungicides which are harmful for human health and causes to much hard currency.

Acknowledgment
The authors are highly thankful to the Division of Biochemistry, Faculty of Basic Sciences, SKUAST-J, for providing necessary facilities in carrying out the research work.

Conflict of Interest
None

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