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Determination of antioxidant activity in overexpressed MPK3 transgenic *Brassica juncea* for induction of defense against Alternaria blight disease

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Abstract
Alternaria blight causes a highly destructive disease in *Brassica juncea* (L.) Czern and Coss (Indian mustard) which leads to significant yield losses. Studies on the *Brassica juncea* MPK3 gene have implicated that MPK3 play a crucial role in plant defense mechanism and involve in disease resistance against Alternaria blight. In this study, the activity of oxidative enzymes was determined in transgenic *B. juncea* (*Bj*V5) plants to assess tolerance against Alternaria blight disease. Ascorbate peroxidase (APX), Guaiacol peroxidase activity (GPOX) and proline content were high in transgenic line (*Bj*V5) of *B. juncea*. Less lipid peroxidation was found in the transgenic plant. Higher reactive oxygen species (ROS) scavenging enzymes in a transgenic plant may have potential to protect against Alternaria blight disease.

Keywords: *Brassica juncea*, MPK3, Alternaria Blight, APX, GPOX and ROS

Introduction
Brassica juncea (L.) Czern and Coss (Indian mustard) is an economically prodigious oilseed crop. It has contained long multi unsaturated omega 3 fatty acid, linoleic and linolenic fatty acid. This crop is exposed to various biotic and abiotic stresses. Major yield loss and reduced crop production of the mustard crop are due to Alternaria blight disease caused by fungal pathogen *i.e Alternaria brassicae*. Alternaria blight is the most damaging and widespread fungal disease of Brassica genotype [1]. Chlorotic toxin like destruxine B produced by a fungal pathogen and plays a role in defense system [2]. However, there is no source of transferable resistance have been found in *B. juncea* cultivar [1]. The Transgenic approach is, therefore alternative methods to developed highly resistance variety of *B. juncea*.
Studies of plant-pathogen interactions have been reported that defense responses against microbes/pathogens are modulated by a complex network of interconnecting signaling pathways in which the plant signal molecules salicylic acid (SA), jasmonate (JA), and ethylene (ET) play an important role against biotic/ abiotic stresses[3,4,5]. Fungicide base disease control is expensive and can be ineffective due to the lack of suitable forecasting methods. Therefore, there is a need to developed highly resistance plant variety in response to fungal pathogen through genetic engineering technology. Genetic manipulation put more and more emphases on genetic resistance against biotic and abiotic stresses [6]. Plants have evolved an effective basal defense system to detect and minimize the pathogen growth. Stress-induced ROS accumulation is counteracted by enzymatic antioxidant systems that include a variety of scavengers, such as Superoxide dismutase (SOD), Ascorbate peroxidase (APX), Guaicol peroxidase (GPOX) and Catalase (CAT) [7]. These antioxidant activities play an important role in maintaining the steady-state levels of reactive oxygen species during pathogenesis and provide defense against the pathogen. Therefore, the present investigation was determined the antioxidant activity in transgenic (*Bj*V5) and wild *B. juncea* var. Varuna.

Materials and Methods
The seeds of transgenic *B. juncea* (*Bj*V5) were obtained from plant stress lab, MBGE and the wild seed of *B. juncea* var. Varuna obtained from crop research center (CRC), GB Pant University of Agriculture &Technology, Pantnagar. Seeds were sown in sterilized soil mix containing a mixture of soil, sand, and vermicompost in the ratio of 2:1:1 and maintained under an appropriate condition with 22 ±1°C, 16/8 hours (light/dark) photoperiod in a transgenic glass house. Pure *Alternaria brassicae* spores were collected from CRC, Pantnagar and spores suspension were inoculated on 45 days old transgenic and wild plants with 80-90% relative humidity and temperature of 20-22ºC for the development of the symptoms. After the inoculation of the pathogen, sampling was done at different time intervals viz. Control, 15 min

1 hr, 6 hr,1 day, Early (5 days after inoculation), Middle (8 days after inoculation) and Late (11 days after inoculation). Total protein was isolated from 0.2 gm of fresh leaf samples homogenized with 50 mM phosphate buffer saline (PBS). Proteins concentration was measured by Bradford method [8].

Catalase (EC 1.11.1.6)

Catalase activity was determined by the method of Dhindsa *et al*, (1981)[9]. The reaction mixture was used in a final volume of 1 ml comprised 50 mM sodium phosphate buffer (pH7.0) with 50 µl of enzyme extract. The enzyme activity was determined by adding 35 µl H_2O_2 at an interval of 5 seconds for 1 min by measuring the decrease in absorbance at 240 nm. The catalase activity was measured using extinction coefficient 39.4 M-1cm-1.

Ascorbate Peroxidase (APX) (EC 1.11.1.1)

APX activity was determined by the method given by Nakano and Asada (1981) [10]. The oxidation of ascorbate was measuring the decrease in absorbance at 290 nm. The reaction volume of 1 ml was used with 910µl of 50 mM sodium phosphate buffer, 55 µl enzyme extract and 35 µl of the H_2O_2 solution. The rate constant was calculated using the extinction coefficient of 2.8 mM-1cm-1.

Guaiacol peroxidase activity (GPOX) (EC 1.11.1.7)

GPOX activity was determined by the method of Plewa *et al*, (1991) [11]. The conversion of guaiacol to tetraguaiacol was measured at 470 nm. The reaction mixture of 1 ml was contained 935 µl phosphate buffer, 25µl of enzyme extract, 15µl guaiacol, and 25µl H_2O_2. The reaction was initiated by adding H_2O_2 and rate of change in absorbance were recorded at 470 nm for 1 min at an interval of 5 seconds. Peroxidase activity has been defined as the change in absorbance min-1mg-1protein.

Malonaldehyde (MDA)

The level of lipid peroxidation was determined in term of malondialdehyde (MDA) content by the method of Heath and Packer (1968) [12]. For the extraction of malondialdehyde, 0.2 gm of leaf tissue was homogenized in 0.1% TCA. The homogenate was centrifuged at 10,000 rpm for 10 min at room temperature. To 0.3 ml aliquot of the supernatant, 1.2 ml of 0.5% of thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95 ºC for 30 min and cooled in an ice bath. The Mixture was centrifuged at 10000 rpm for 10 min at room temperature and the absorbance of the supernatant was measured at 532 and 600 nm. MDA contents were calculated by extinction coefficient of 155 mM-1cm-1 and results were expressed as µmole malondialdehyde per gram of fresh weight.

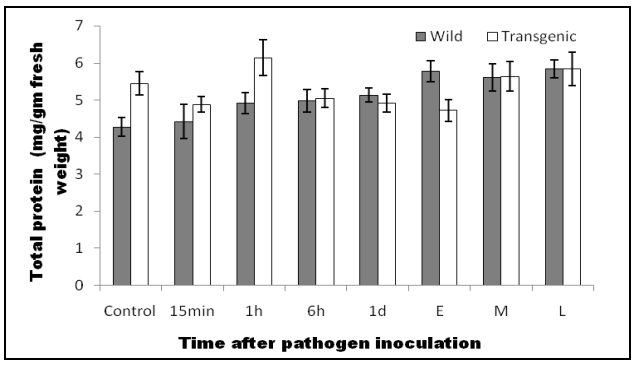
Proline content

The extraction of proline content was measured by the method of Bates *et al*, (1973) [13]. 0.2 gm of fresh leaf sample was homogenized with 2ml of 3% aqueous sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min. The supernatant was estimated as proline content. The reaction mixture consisting of 1 ml of supernatant, 1 ml of acid ninhydrin and 1 ml of glacial acetic acid were mixed and boiled at 100°C for 1 hour. After terminated by reaction in an ice bath, the reaction mixture was extracted with 4 ml toluene, mixed vigorously for 15-20 seconds. An absorbance of the reaction mixture was measured at 532 nm. The amount of protein was expressed in µg per gram of fresh weight using the standard curve of known concentration of proline.

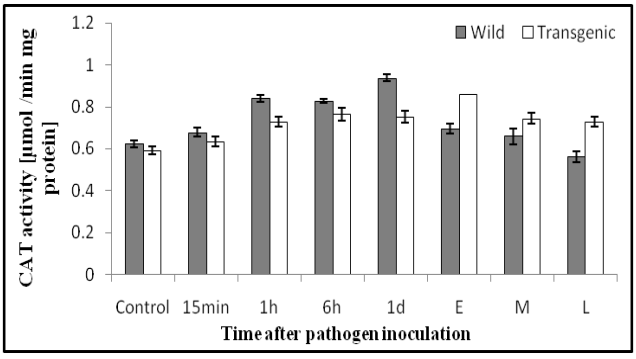
Statistical analysis

All experiments were repeated in triplicates and the results were expressed as mean ±SE. All measurements within transgenic and wild plants were statistically compared using an analysis of variance (ANOVA) at the 5% probability level ($p \leq 0.05$) with Graph Pad Prism version 5.01, La Jolla California USA.

Results



A.



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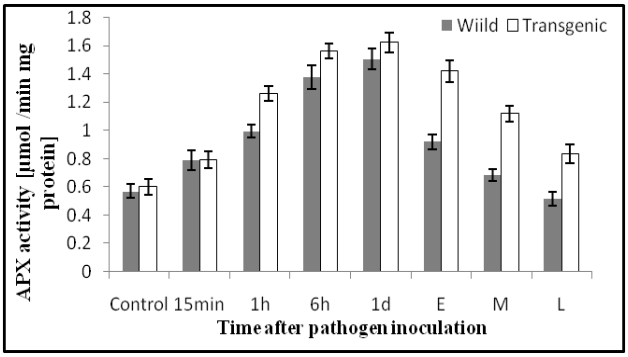

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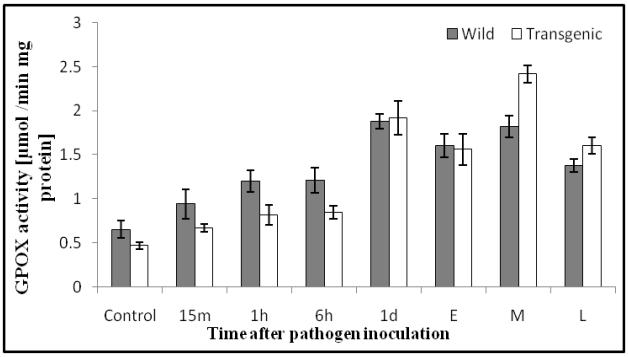

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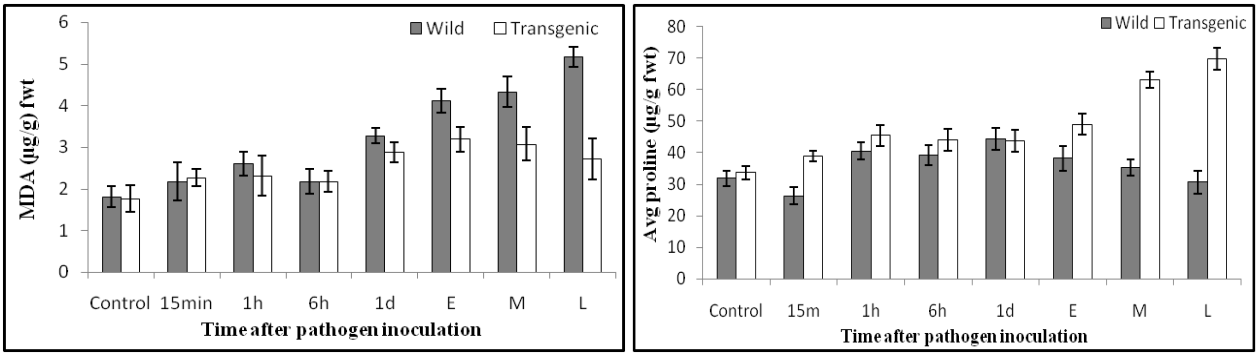

E. F.

Fig 1: Antioxidant components in wild and Transgenic (*Bj*V5) *Brassica juncea* at different time intervals i.e. Control, 15 minute (15m), 1 hour (1h), 6 hour (6h), Early (E), Middle (M) and Late (L) during pathogenesis of *Alternaria brassicae*; (A) Total proteins in mg per gram of fresh weight, (B) Catalase activity, (C) APX activity, (D) GPOX activity, (E) MDA content and (F) proline content. All values were used in means of triplicate measurements ± SE in both transgenic and wild plants. Statistical significance was determined by using an analysis of variance ($p \leq 0.05$).

Antioxidant enzymes activity

Noninoculated leaf extract of both transgenic and wild plant had an almost similar activity of catalase. In a later stage of pathogenesis, the activity of catalase was significantly high in transgenic plants which might be caused by tolerance against Alternaria blight disease (Fig. 1B). After inoculation of a pathogen on the plant in transgenic glass house under controlled condition, both wild and transgenic plants were shown significant increased APX content up to 1 day after infection. The APX content was high in the transgenic plant then the wild plant. This high APX content in transgenic line might be a cause of tolerance against the pathogen (Fig. 1C). In a later stage of pathogenesis, the APX content was declined in wild *B. juncea* and disease progression inaugurated in wild plant. The GPOX activity was significantly higher in the wild plant then the transgenic up to 6 hours after pathogen inoculation. In the later stage of infection, the GPOX activity was much high in transgenic lines of *B. juncea* (Fig. 1D). This increased activity of GPOX may be involved in the induction of defense response against Alternaria blight disease.

MDA

The MDA content was used for the degradation of lipid peroxidation. Normal healthy plant of both transgenic (*Bj*V5) and wild plants was treated as a control, where MDA content was almost equal. However the pathogen was inoculated onto the plant leaf, the MDA content was observed to be increased in wild plant (Fig. 1E). Less content of MDA in pathogen-inoculated transgenic plant shown less lipid peroxidation which has indicated that transgenic plant was immune to the pathogen.

Proline

The proline content increased markedly measured in the transgenic plant over the wild plant. In fact, proline content was significantly high in the transgenic plant up to a later stage of pathogen infection (Fig. 1F). Increasing trend of proline in transgenic plant shows the tolerance against pathogen and provide defense against Alternaria blight disease.

Discussion

Indian mustard is an essential oilseed crop in India. The productivity of mustard is declined year by year due to various biotic and abiotic factors. Alternaria blight disease is a most dominating disease of the mustard family. We have developed transgenic lines of *Brassica juncea* by constitutive expression of MPK3 gene. Moreover, many researchers provide that MPK3/MPK6 cascade is also involved in defense gene activation, biomass production, reactive oxygen species (ROS) generation, and hypersensitive response-like cell death [14, 15]. In spite of the fact that ROS provides effective resistance against pathogens, a few pathogens may involve in the production of ROS for own advantage. For instance, necrotrophs seem to promote ROS production in the affected tissue to induced programmed cell death for induction of defense response[16]. In the present investigation, we employed the antioxidant activity in transgenic (BjV5) and wild *B. juncea*. Various antioxidative defense mechanisms are scavenged ROS molecules and provide defense against biotic and abiotic factors [17]. APX activity was increased in a transgenic plant, which scavenged the ROS from cells and induced defense response against Alternaria blight. The comparable outcome was also reported by Mitler *et al*, (1999) [18], who revealed that the transgene expression of cAPX in the tobacco plants in an improved PCD because of necrotrophic pathogen attack. The higher activity of GPOX and high proline content in the transgenic plant might be involved in the induction of defense responsive genes toward the pathogen. The study also supported that significantly increased GPOX activity in transgenic tomato plants provides resistance against *Alternaria solani* pathogen [19]. GPOX activity was also significantly increased in necrotrophic pathogen inoculated resistant plant as compared with susceptible plant [20]. Lipid peroxidation was seen in the form of MDA content. Transgenic plants were shown less lipid peroxidation during the pathogenesis of *Alternaria brassicae* pathogen upto the later stage of pathogenesis. The low level of MDA in the cell, an indirect indicator of lipid peroxidation [21]. This study clearly demonstrated that overexpression of MPK3 in transgenic (*Bj*V5) *B. juncea* plant is modulated the MAPK machinery and promote increased antioxidative enzymes activity for scavenged ROS and induced defense against *Alternaria brassicae* pathogen.

Conclusions

In the finding of this study suggest that the transgenic (*Bj*V5) plant of *B. juncea* might be developed defense against the pathogen of *Alternaria brassicae*, may have potential to protect against Alternaria blight disease. For a better understanding of plant-pathogen interaction, we need to further study on protein-protein interaction to know about novel protein, which involved in the pathogenesis of Alternaria blight disease.

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Conflicts Of Interest

The authors declared no conflict of interest.

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