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Research article

Histo-chemical and biochemical analysis reveals association of *er1* mediated powdery mildew resistance and redox balance in pea

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ABSTRACT

Powdery mildew caused by *Erysiphe pisi* is one of the important diseases responsible for heavy yield losses in pea crop worldwide. The most effective method of controlling the disease is the use of resistant varieties. The resistance to powdery mildew in pea is recessive and governed by a single gene *er1*. The objective of present study is to investigate if *er1* mediated powdery mildew resistance is associated with changes in the redox status of the pea plant. 16 pea genotypes were screened for powdery mildew resistance in field condition for two years and, also, analyzed for the presence/absence of *er1* gene. Histochemical analysis with DAB and NBT staining indicates accumulation of reactive oxygen species (ROS) in surrounding area of powdery mildew infection which was higher in susceptible genotypes as compared to resistant genotypes. A biochemical study revealed that the activity of superoxide dismutase (SOD) and catalase, enzymes involved in scavenging ROS, was increased in, both, resistant and susceptible genotypes after powdery mildew infection. However, both enzymes level was always higher in resistant than susceptible genotypes throughout time course of infection. Moreover, irrespective of any treatment, the total phenol (TP) and malondialdehyde (MDA) content was significantly high and low in resistant genotypes, respectively. The powdery mildew infection elevated the MDA content but decreased the total phenol in pea genotypes. Statistical analysis showed a strong positive correlation between AUDPC and MDA; however, a negative correlation was observed between AUDPC and SOD, CAT and TP. Heritability of antioxidant was also high. The study identified few novel genotypes resistant to powdery mildew infection that carried the *er1* gene and provided further clue that *er1* mediated defense response utilizes antioxidant machinery to confer powdery mildew resistance in pea.

1. Introduction

Pea (*Pisum sativum* L.) is one of the important grain legume crop worldwide mainly used for consumption as green vegetables and dry seeds (Katoch et al., 2010). Pea crop is cultivated in 7.3 million hectares with total production 600 metric tons globally (FAOSTAT, 2013). The productivity of pea is affected by many diseases and insect-pests (Kraft and Pfleger, 2001). Among the various diseases affecting pea, powdery mildew, caused by *Erysiphe pisi*, is of great significance. It affects all the green parts of the plant and cause yield loss 25–50% (Warkentin et al., 1996). This disease adversely affects the biomass and yield of plants mainly by reducing a number of pods per plant, seeds per pod and plant height (Gritton and Ebert, 1975). The disease spreads rapidly during the dry weather when the nights are cooler (Reilling, 1984). The biological cycle of *E. pisi* includes germination of conidia (asexual spores) or ascospores (sexual spores), a formation of appressorium and haustorium, development of colonies epiphytically on the host epidermis and production of new spores for repeated infection (Pavan et al., 2011).

The most efficient and ecologically sound strategy to manage the disease is the use of resistant cultivars. The pea cultivar Xucai 1 has shown high resistance to *E. pisi* under greenhouse and field conditions (Sun et al., 2015). The resistance to powdery mildew in pea is governed by a single recessive gene '*er1*' (Harland, 1948; Pierce, 1948). Later, two other genes for resistance named *er2* and *Er3* (Heringa et al., 1969; Fondevilla et al., 2007) have also been

Abbreviations: SOD, Superoxide dismutase; MDA, Malondialdehyde; CAT, Catalase; TP, Total phenol; AUDPC, Area Under Disease Progress Curve; DAB, 3,3-diaminobenzidine; NBT, Nitro blue tetrazolium; ROS, Reactive Oxygen Species; EU g^{-1} FW, enzyme units per gram fresh weight; TCA, Trichloro acetic acid; TBA, Thiobarbituric Acid; hai, hours after inoculation.

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described. Gene *er1* is widely used in pea breeding programme and provides complete to partial resistance depending on the locations (Heringa et al., 1969; Tiwari et al., 1997a,b; Fondevilla et al., 2006). Resistance conferred by gene *er1* is reported to be stable as it causes an effective barrier to the pathogen penetration (Fondevilla et al., 2006). There are reports that plant resistant gene also works with the complex defense system against fungal infections (Dangl and Jones, 2001). These complex responses leads to the rapid generation of reactive oxygen species (ROS), which includes superoxide anion (O_2-), hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2) (Patykowski and Urbanek, 2003). These ROS function either directly in the establishment of defense mechanisms or indirectly through synergistic interactions with other signaling molecules (Bolwell and Daudi, 2009). Antioxidants, such as ascorbate, glutathione and phenolic compounds (Foyer, 2001) and ROS-scavenging enzymes i.e., SOD and CAT (Bowler et al., 1991, Asada, 2006) and lipid peroxidation are primarily involved in maintaining the redox balance of cells under various stresses. Complex arrays of detoxification mechanisms have been selected in plants against ROS accumulation and toxicity. The excess ROS production in the cell leads to oxidative damage, thereby promoting lipid peroxidation, damaging macromolecules such as pigments, proteins, nucleic acids and lipids (Apel and Hirt, 2004). To reduce the toxic effect of oxidative damage, plants activates antioxidative system such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX) and catalase (CAT) (Mittler, 2002) to modulate the cytotoxic effects of these free radicals.

Thordal-Christensen et al. (1997) detected H_2O_2 accumulation in developing papillae and surrounding cell wall appositions in the incompatible interaction of barley–powdery mildew. They demonstrated these phenomena using DAB (3, 3-diaminobenzidine) staining and indicated that the accumulation of H_2O_2 always occurred beneath the infection sites during the barley-powdery mildew interactions. The generation of ROS at the interaction sites in near isogenic lines (NILs) during infection by *Blumeria graminis f. sp. hordei* was studied by Huckelhoven et al. (1999). Similarly, Kirik et al. (1974) observed high peroxidase and polyphenol oxidase activity in powdery mildew resistant pea cultivars. In an earlier study by Kalia and Sharma (1988) revealed higher levels of phenolic and phenol-enzymes in resistant cultivars than the susceptible after the infection of powdery mildew. The previous study on resistance to powdery mildew was based on disease reaction without prior knowledge of the presence of '*er1*' gene. There is no information about the regulation of defense molecule(s) and histo-chemical events in presence and absence of the *er1* gene in a different genotypic background. Hence, the present investigation was carried out to study *er1* gene induced histological and biochemical parameters associated with powdery mildew resistance in pea.

2. Material and methods

2.1. Plant material and experimental design

Sixteen pea genotypes obtained from Department of Genetics and Plant Breeding, Banaras Hindu University, Varanasi were evaluated under field conditions at Agricultural Research Farm (North Eastern Plain zone, India, 25.2° N and 83.0° E) during two consecutive cropping seasons 2013–14 and 2014–15.

The characteristics features of these genotypes are given in Table 1. These genotypes were planted in a randomized block design (RBD) with three replications. Each genotype were grown in a 2 m long row with inter and intra row spacing of 30 and 10 cm, respectively. For uniform disease development and subsequent spread of pathogen, an infector row comprising one row of susceptible pea genotype (PG-3) was planted after every 5th row in the plots. The recommended agronomic practices were adopted to ensure good crop.

2.2. Pathogen inoculation

Single colony based inoculum of *E. pisi* was maintained on susceptible pea genotype PG-3. Plants were inoculated with the pathogen by tapping the conidia on leaves with the help of camel's hair brush on the young leaves of pea plants (Lim, 1973). For screening of pea genotype powdery mildew spores were collected from the susceptible checks and inoculated the test genotypes manually (Nisar et al., 2006).

2.3. Scoring of disease severity

Disease severity was visually scored on individual plants using 0 to 9 scale (Warkentin et al., 1995). First scoring was done when symptoms of powdery mildew appeared on all the genotypes, second and third readings were taken at 7 days interval.

Area under disease progress curve (AUDPC) was calculated as per the formula of Shaner and Finney (1977):

$$\text{AUDPC} = \sum_{i=1}^{n} [\{Yi + (Yi+1)/2\{(ti+1) - ti\}]$$

Where, Yi = disease level at time ti.
(ti+1)-ti = Time (days) between two disease scores.
n = no. of observations (score)

2.4. Genotyping for the presence of resistance gene er1

The presence or absence of *er1* gene in the pea genotypes was ascertained by using a gene based marker i.e., *PsMlo2* (Mohapatra et al., 2016). The presence of *er1* gene was determined by an amplification product of ~900 bp in resistant whereas; susceptible genotypes were marked by the presence of an amplification product of ~1250 bp.

2.5. Histo-chemical studies

All 16 genotypes (Table 2) were taken for DAB and NBT staining based on time of infection of powdery mildew fungi on pea leaves. The leaf samples were collected from both the resistant and susceptible genotypes before and after inoculation of the pathogen (0 h, 24 hai, 48 hai and 72 hai).

2.5.1. Localization of H_2O_2

Localization of H_2O_2 generation in the powdery mildew infected leaf samples was done using 3, 3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997). Three cuts of 1 cm^2 leaves from 5 tagged plants of each 16 genotypes showing powdery mildew infection were collected and submersed in a solution containing 5 ml of DAB staining solution (1000 μl/ml) in 5 ml tubes. The tubes were placed on a standard laboratory electrical shaker for 7–8 h to allow the uptake of DAB and its reaction with H_2O_2 at 80–100 rpm. After staining of the leaves, DAB solution was replaced by bleaching solution (Ethanol: Acetic acid: Glycerol = 3:1:1) and the tubes were then placed carefully in boiling water bath (90–95 °C) for 15 min to bleach out the chlorophyll. After 15 min of boiling, bleaching solution was replaced by fresh bleaching solution and allowed to