Host defense responses and genetics of inheritance of resistance to powdery mildew (*Golovinomyces orontii*) in sunflower (*Helianthus annuus* L.)

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for the award of the degree of

DOCTOR OF PHILOSOPHY

by

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Declaration

This is to declare that I, K. Prathap Reddy, have carried out the research work embodied in the present thesis entitled **"Host defense responses and genetics of inheritance of resistance to powdery mildew (*Golovinomyces orontii*) in sunflower (*Helianthus annuus* L.)**" and submitted for the degree of Doctor of Philosophy under the supervision of Dr. M. Sujatha, Principal Scientist, ICAR-Indian Institute of Oilseeds Research, Hyderabad and Prof. P.B. Kirti, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad. I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma from any University. A report on plagiarism statistics from university Librarian is enclosed

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This is to certify that, the thesis entitled **"Host defense responses and genetics of inheritance of resistance to powdery mildew (*Golovinomyces orontii*) in sunflower (*Helianthus annuus* L.)"** is based on the results of the work done by **Mr. K. Prathap Reddy** for the degree of Doctor of Philosophy under our supervision. This work presented in this thesis is original and has not been submitted for any degree or diploma of any other University. A report on plagiarism statistics from university Librarian is enclosed.

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1	Research Methodology	4	Pass
2	Frontiers in Life Sciences	2	Pass
3	Seminar	1	Pass
4	Lab Work	8	Pass
5	Scientific Writing	1	Pass

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Dedicated to the Almighty, my family and my teachers

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(K. Prathap Reddy)

Abbreviations

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
AUDPC	Area under the disease progress curve
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-ropanesulfonate
CTAB	Cetyltrimethylammonium bromide
D/F	Days to flowering
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DPI	Days Post Infection
DSI	Disease Severity Index
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
FDH	Formate dehydrogenase
GOBP	Gene Ontology Biological Process
GOCC	Gene Ontology Cellular Component
GOMF	Gene Ontology Molecular Function
GPX	Glutathione peroxidase
GR	Glutathione reductase
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HR	Hypersensitive reaction
HSFs	Heat-shock transcription factors
HSP	Heat shock proteins
ID	Interspecific derivative
ITS	Internal transcribed spacer
KCl	Potassium chloride
KEGG	Kyoto Encyclopedia of Genes and Genomes
KV	Kilo volt
LC-MS/MS	Liquidchromatography–massspectrometry/massspectrometry
LOD	Logarithm of odds
$MgCl_2$	Magnesium chloride
MS	Mass spectrometry
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS-LRR	Nucleotide-binding site leucine-rich repeat
NBT	Nitro blue tetrazolium
OD	Optical density

PANTHER	Protein ANalysis THrough Evolutionary Relationships
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PI	Plant Introduction number
POX	Peroxidase
PVP	Polyvinylpyrrolidone
QTL	Quantitative trait locus
RH	Relative humidity
RILs	Recombinant Inbred Lines
RNA	Ribonucleic acid
ROIs	Reactive Oxygen Intermediates
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SSR	Simple sequence repeats
TAE	Tris base, acetic acid and EDTA
TCA	Trichloroacetic acid
UPLC	Ultra-Performance Liquid Chromatography

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Chapter 1

Introduction

Sunflower

Sunflower belongs to the family Asteraceae and subfamily Asteroidae, tribe *Heliantheae*, subtribe *Helianthineae* (Panero and Funk, 2000). Sunflower is an annual cultivated crop and cultivated hybrid plant with nonbranched, whereas wild species are with dominant branching. Both annuals and perennials exist in the wild species. The basic chromosome number of the genus *Helianthus* was n=17 and contains diploids (2n=2x=34), tetraploid (2n=4x=68), and hexaploid (2n=6x=102) species. Among the sunflower species, the 14 annual species are all diploid, and 37 perennials species includes 27 diploid, 4 tetraploid, 6 hexaploid and 4 mixiploid species. The sunflower genome size was estimated to be 3.6 Gb (Price et al., 2000).

Sunflower is the second largest hybrid crop that was cultivated worldwide after maize and fifth largest vegetable oilseed crop with value over US$ 18.5 billion (FAO Stat, 2017). The economic value of sunflower is mainly derived from the extracted oil (80% value) (Fick and Miller, 1997). The presence of high oleic and saturated fatty acids provides frying functionality and it is preferred in the frying industry. Tocopherol is one of the important natural fat soluble antioxidant that is present in sunflower. Besides its major utility as vegetable oil, it is used in the manufacture of lacques, polyester fills, co-polymers, plasticizers and modified resins (Seiler and Jan, 2010). It is also used in the manufacture of soaps and detergents (Mishra, 2013). It is used as a confectionery, food or snack. Sunflower is a good source of animal feed; it can successfully replace soybean meal in feeding ruminant animals (Seiler and Jan, 2010).

In India, sunflower is one of the major oilseed crops that is grown as a main source as cooking oil and sunflower is grown in 7 major states with an annual production of 0.33 lakh tonnes. Among the states, Karnataka accounts for the 50% share of total production followed by Haryana, Andhra Pradesh, Bihar, Tamilnadu and Uttar Pradesh (Anonymous, 2017). In the year 2007, India was the one of the top ten largest sunflower producers (Seiler and Jan, 2010), but the area under harvest and production were drastically reduced during the last ten Years (Fig 1.1) (FAO stat, 2017). The import of sunflower accounts to 10.6 lakh tonnes, which is the 5th largest commodity that is imported by India. It accounts for 1.2 billion USD import value with its 2nd position among top ten by the country (FAO Stat, 2017).

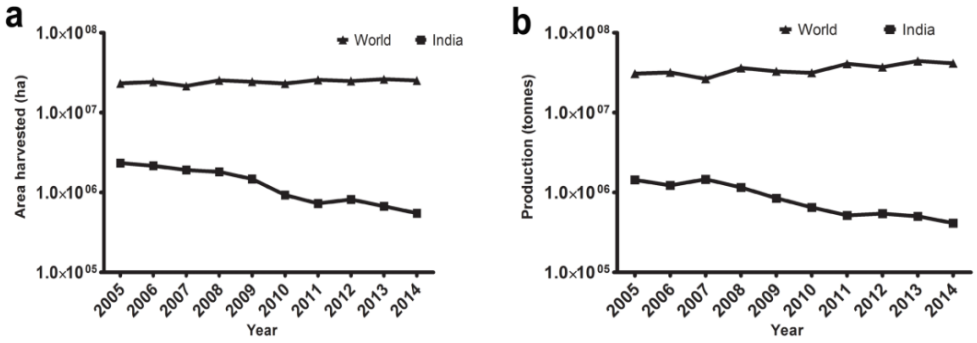

Fig 1.1 a) Area harvested and b) production of sunflower during the last 10 years between world and India (FAO Stat, 2017)

The major constraints for profitable production of sunflower across the globe including India is the vulnerability of the improved cultivars to biotic and abiotic stresses. The major abiotic stress include drougt and salinity. Drought is the major abiotic stress that affects 26% of the arable area of sunflower (Skoric, 2016) and it may cause drastic yield losses if occurs at flowering stage (Rauf, 2008). Upto 30% yield losses were reported due to salinity in sunflower (Hussain and Rehman, 1992). The important diseases that cause significant yield losses in sunflower are downy mildew (Gulya et al., 2013), rust (Shtienberg and Zohar, 1992), chlorotic mottle virus (Lenardon et al., 2001), sunflower necrosis (Bhat and Reddy, 2016), *Alternaria* leaf spot (Carson, 1985), powdery mildew and Phomopis (Roustaee et al., 2000). In India, the major diseases that are prevalent in sunflower growing areas are sunflower necrosis, *Alternaria* leaf spot, powdery mildew and downy mildew (Basappa and Santhalakshmi Prasad, 2005; Reddy et al., 2013). The adverse climate and increased incidence of diseases like powdery mildew, *Alternaria* leaf spot and sunflower necrosis disease had drastically reduced the sunflower area under cultivation and production in India from past 10 years (Fig. 1.1).

Powdery mildew and its economic importance

In the recent past, the severity of incidence of the various diseases became accelerated and hitherto unknown and minor diseases turned out to be the major diseases with regular occurrence. The important diseases that cause significant yield losses in sunflower in India are Alternaria leaf spot, downy mildew, sunflower necrosis, powdery mildew and rust (Kolte, 1985; Basappa and Santhalakshmi Prasad, 2005; Reddy et al., 2013). In India, the disease

scenario has been changing quite rapidly becoming a constant challenge to the breeders and crop protection researchers for deployment of suitable management strategies. The pathogen, *Golovinomyces orontii* (formely *Golovinomyces cichoracerum*/ *Erisiphe cichoracerum*) has been reported to causes powdery mildew disease in in the tropical and sub tropical regions (Fig. 1.2). During the past decade, powdery mildew has become a severe problem and has spread rapidly to all sunflower cultivation regions of the country (Reddy et al., 2013; Sujatha et al., 2015). Powdery mildews have world-wide distribution, but greater intensity on sunflower is reported in the tropical regions (Zimmer and Hoes, 1978; Diaz-Franco, 1980; Gulya et al., 1997; Anyanga and Biruma, 2010). The disease is reported to cause yield losses up to 13% in Mexico (Diaz-Franco, 1980), 25% in USA between 1983 and 1989 (Gulya et al., 1991) and 30-74% in India depending on the disease severity (Dinesh et al., 2010; Sujatha et al., 2015; Madhusudhan et al., 2017).

Fig. 1.2 Powdery mildew infection in sunflower (a & b) Cotyledonary leaf, (c) Young leaf and stem, (d & e) Wild species, (f) Cultivated sunflower (whole plant), (g) Sunflower head, (h & i) Leaves at maturity stage showing infection

Management of powdery mildew in sunflower

In general, fungicides are largely used for management of powdery mildew disease in sunflower. In a field test, the fungcides Bayleton and Tilt controlled the powdery mildew disease in California (Kontaxis, 1988). The fungicides like propiconazole, difenoconazole, carbendazin and hexaconazole were effective against powdery mildew (Dinesh, 2009). The fungicides macozeb and wettable sulphur have significantly reduced the powdery mildew

incidence (Akhileswari et al., 2012). The new chemicals like topas, clone, aerosol, cumulus and sufex gold were found to be superior in controlling powdery mildew disease (Yasir et al., 2016).

However, in the recent past the indiscriminate usage of fungicide has become a serious concern for regulating the usage of fungicides. In the year 2014, the global usage of fungicides accounts 3.6 lakh tones of active ingredients. In the year 2010, India alone accounted for 13,055 tonnes of active ingredients (FAO Stat, 2017). This injudicious use of fungicides had resulted in development of resistance in the pathogen against the fungicides. It raises a serious problem of environmental and health concern, including, water, soil, air pollution. Hence, there is an urgent need for employing resistance breeding progammes by utilization of resistance sources from natural germplasm to combat the problem.

Sources of powdery mildew resistance in sunflower wild species and cultivated germplasm

Previous studies had reported resistance sources in wild sunflowers against powdery mildew (Saliman et al., 1982; Skoric, 1984: Jan and Chandler, 1985; 1988; McCarter, 1993; Acimovic, 1998; Rojas-Barros et al., 2004; 2006; Christov, 2008; Dedic et al., 2012; Reddy et al., 2013). Screening studies reported on identification of resistance sources in cultivated sunflower (Reddy et al., 2013; Kulkarni et al., 2015; Supriya et al., 2016; Suresha et al., 2017). Screening resulted in identification of resistant sources for powdery mildew. There is a need to understand the infection process for deploying suitable resistant sources against the powdery mildew.

Characterization of powdery mildew resistant plant material and host defense against powdery mildew- a brief review on other crops

The combined use of classical morphological (light microscopy) and SEM analyses had successfully compared the morphological characteristics of powdery mildew in many crops (Cook et al., 1997; Kiss et al., 2001; Cook and Braun, 2009; Cook et al., 2011). These techniques are widely used in studying the infection process of pathogen into host and their interactions (Celio and Hausheck, 1998; Adam and Somerville, 1998; Adam et al., 1999; Li et al., 2006; Micali et al., 2008; Cook et al., 2011). Plant-pathogen interaction, particularly in the case biotropic parasites are caused by specific interactions between the pathogen *avr* (avirulence) gene loci and alleles of the corresponding R-locus. The presence of both the genus is essential for disease resistance, whereas failure of either results in disease (Flor,

1971). Several studies have reported the identification of many R genes from model and crop species (Flor, 1971; Bent, 1996; Ellis, 2000). The interaction between powdery mildew and host results in a cascade of complex cellular processes involved by both the host and pathogen. Plant deploys various mechanisms to combat the pathogen attack. The determinants of resistance have been studied using genomic, transcriptomic, biocheimical and proteomic techniques (Michelmore, 2000; Rampitsch and Bykova, 2012; Lodha et al., 2013; Thorpe et al., 2014).

One of the important responses from a host to its biotrophic fungal counterpart is the hypersensitive reaction (HR). A pathogen attack triggers the generation of reactive oxygen intermediates (ROIs) by a variety of enzyme systems (Bolwell et al., 2002; Babitha et al., 2004; Deepak et al., 2006; Zimmermann et al., 2006; Bindschedler et al., 2006; Cona et al., 2006; Carter et al., 2007; Amirsadeghi et al., 2007). ROIs are involved in various signalling pathways in defense mechanisms such as triggering of the HR, accumulation of phytoalexins and a number of other defense response genes. A higher concentration of ROIs has the potential to cause oxidative damage by reacting with bio molecules, whereas they act as powerful signalling molecules that are involved in the regulation of plant growth and development as well as priming acclimatizing responses to the stress stimuli, when they are below the threshold levels (Bowler and Fluhr, 2000; Foyer and Noctor, 2009; Yadav et al., 2016). The synergy between the antioxidant enzymes and redox metabolites modulates the steady-state levels of superoxide radicals and hydrogen peroxide.

The major ROIs scavenging agents existing in the host cells include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Asada and Takahashi, 1987; Willekens et al., 1997). The balance in SOD, APX and catalase is crucial for maintaining the superoxide radicals and hydrogen peroxide (Bowler et al., 1991; Martinez et al., 1998; Blee et al., 2001; Hilaire et al., 2001; ShivaKumar et al., 2003; Passardi et al., 2004). The role of these enzymes in different host-disease responses has been adequately demonstrated. Transcripts of sunflower antioxidant scavengers of the SOD and GPX families are differentially expressed in response to downy mildew and treatment with stress signalling factors (Herbette et al., 2003). The higher levels of APX in powdery mildew resistant wheat compared to the susceptible line contributed to the elimination of ROI (Chen et al., 2006). Up regulation of APX gene was reported in barley leaves after inoculation with powdery mildew (Burhenne and Gregersen, 2000). Over expression of the

glutathione reductase gene in powdery mildew susceptible wheat enhanced the resistance of the host to *Blumeria graminis* (Chen et al., 2007). The powdery mildew resistance in oat genotypes was correlated with increased SOD concentration in the leaf (Venacker et al., 1998).

ROIs involved in various signaling pathways for defense mechanisms such as triggering of the HR, accumulation of phytoalexins and a number of other defense response genes. The recognition ROIs in plants take place by three mechanisms (Mittler et al., 2004). These include unidentified receptor proteins; redox-sensitive transcription factors such as NPR 1 or heat-shock transcription factors (HSFs); and direct inhibition of phosphate (Apel and Hirt, 2004; Mittler et al., 2004; Neil et al., 2002). Phosphorylation of proteins, changes in ion flux or oxidative burst, leading to either HR or defense gene expression, or both are important events taking place after pathogen infection (Jabs et al., 1997; Chandra et al., 1996; Lamb and Dixon, 1997; Sasabe et al., 2000). The major ROIs scavenging mechanisms that exist in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Asada and Takahashi, 1987; Bowler et al., 1992; Willekens et al., 1997).

In the past few years, plant proteome analysis has significantly exploded our knowledge in several physiological and stress related mechanisms, including plant-pathogen interactions (Nat et al., 2007; Quirino et al., 2010; Delavnois et al., 2014; Gupta et al., 2015). Proteomics, a comprehensive and quantitative analysis of proteins that are expressed in a given organism under a given situation, provides an understanding of the biological process that cannot be obtained by genomics or transcriptomic approaches (Wolters et al., 2001; Gerster et al, 2010). Advances in mass spectrometry and progress in bioinformatics have accelerated the proteomic approaches in studing plant-pathogen interactions (Quirino et al., 2010).

In sunflower, proteomes were analyzed for abiotic stresses like heavy metal stress (Walliwalagedara et al., 2010; Lopes et al., 2015), drought acclimation (Fulda et al., 2011), cold acclimation (Balbuena et al., 2011), heterosis (Mohayeji et al., 2014; Alireza, 2014) and seed quality traits (Baudet and Mosse, 1997; Hajduch et al., 2007; Furse et al., 2013). One study reported the proteomic analysis of sunflower in response to *Sclerotinia sclerotiorum* fungus infection (Akram et al., 2015). Different donors conferring resistance to powdery mildew infection showed different levels of resistance. Proteomic analysis would provide information related to the candidate proteins and protein pathways involved in the resistance

mechanism. Studies with barley (Godfrey et al., 2009; Noir et al., 2009; Bindschedler et al., 2009; 2011), wheat (Li et al., 2011; Oberhaensil et al., 2011; Wang et al., 2012; Liu et al., 2013), pea (Curto et al., 2006) and grapevine (Marsh et al., 2010) have contributed significantly in defining the specific function of proteins involved in powdery mildew interaction with these crops.

Inheritance of resistance and molecular mapping of powdery mildew resistance genes in sunflower

The methods employed for breeding for resistance to diseases in sunflower can be categorized into two broad types *viz.* resistance controlled by major genes and quantitative inheritance. Very few studies were reported on inheritance of resistance for powdery mildew in sunflower. Inheritance of resistance to powdery mildew follows both types of resistance. Jan and Chandler (1988) had reported contribution of a partially dominant gene for resistance. Two gene controlled inheritance was proposed (Rojas-Barros et al., 2006). Christov (2008) reported the existence of both types of inheritance in wild *Helianthus* species. In one type, the inheritance was controlled by a single dominant gene, whereas in the other, it was polygenic. Similarly existence of both single and polygenic inheritance was reported by Naggayya (2013). All these studies are based on either low number of individuals in the population (Jan and Chandler, 1988; Rojas-Barros et al., 2006; Christov, 2008) or considering the irregular disease frequency distribution.

Against this background and keeping in view the importance of powdery mildew disease on sunflower in India, the present investigation has been undertaken with the following objectives.

Objectives

- Identification of the genus causing powdery mildew in sunflower in India.
- Development of a new scoring scale for powdery mildew disease and identification of resistance sources in cultivated and wild sunflowers.
- Histopathological study of the powdery mildew infection process in sunflower and host defense responses during powdery mildew infection in sunflower.
- Differential proteomic analysis in sunflower - powdery mildew infection process.
- Development of RIL population for studying the inheritance of resistance to powdery mildew and molecular mapping of powdery mildew resistance genes in sunflower.

Chapter 2

Review of Literature

2.1. Sunflower

Sunflower (*Helianthus annuus* L.) is the second largest hybrid crop that is cultivated worldwide after maize and fifth largest vegetable oilseed crop with value over US$ 18.5 billion (FAO Stat, 2014). The largest producers of sunflower are Ukraine, Russia, China, Romania, Argentina, and Bulgaria (Table 2.1). Global production grew steadily in the last 25 years and the Food and Agriculture Organization (FAO) anticipated that by the year 2050, total world production of sunflower would be close to 60 million tons (Brando and Sarquis, 2012). Sunflower production is limited by both abiotic and biotic stresses and production is unpredictably changing from areas of high productivity to marginal areas with lower yield potential (Seiler, 2008).

Table 2.1. Top 20 countries of sunflower production (FAO Stat, 2017)

S. No	Country	Production (tonnes)
1	Ukraine	10133750
2	Russian Federation	8475346
3	China, mainland	2380000
4	China	2380000
5	Romania	2189309
6	Argentina	2063410
7	Bulgaria	2010668
8	United Republic of Tanzania	1721875
9	Turkey	1637900
10	Hungary	1597250
11	France	1584190
12	United States of America	1004630
13	Spain	952986
14	South Africa	832000
15	Republic of Moldova	547497
16	Kazakhstan	512765
18	Serbia	509250
19	Myanmar	456004
20	**India**	**415000**

The major constraints for profitable production of sunflower across the globe including India is the vulnerability of the improved cultivars to biotic and abiotic stresses. The major Abiotic stress include drougt and salinity. Drought is the major abiotic stress that affects 26% of the arable area of sunflower (Skoric, 2016), it might cause drastic yield losses

if occurs at flowering stage (Rauf, 2008). Upto 30% yield losses were reported due to salinity in sunflower (Hussain and Rehman, 1992). Among the important diseases that cause significant yield losses in sunflower are downy mildew (Gulya et al., 2013), rust (Shtienberg and Zohar, 1992), chlorotic mottle virus (Lenardon et al., 2001), sunflower necrosis (Bhat and Reddy, 2016), *Alternaria* leaf spot (Carson, 1985; Balasubrahmanyam and Kolte, 1980), powdery mildew and Phomopis (Roustaee et al., 2000). In India, sunflower is grown in 7 major states with an annual production of 0.33 million tonnes from a cultivated area of 0.37 million ha (Anonymous, 2017). The major producers of sunflower are Karnataka followed by Haryana and Andhra Pradesh (Table 2.2). The major diseases that are prevalent in sunflower growing areas of India are sunflower necrosis, Alternaria leaf spot, powdery mildew and downy mildew (Basappa and Santhalakshmi Prasad, 2005; Reddy et al., 2013). In the recent past, the severity of incidence of the various diseases became accelerated and hitherto unknown and minor disease turned out to be the major diseases with regular occurrence. Among the important diseases that cause yield losses in sunflower are downy mildew, sunflower necrosis, rust, *Alternaria* leaf spot and powdery mildew (Allen et al., 1981; Carson, 1985; Shtienberg and Zohar, 1992).

Table 2.2. Three largest producing states of sunflower during 2015-2016 (Anonymous, 2017)

S. No	State	Production (million tonnes)	% Share in all India production
1	Karnataka	0.17	50.00
2	Haryana	0.04	11.52
3	Andhra Pradesh	0.02	06.97
4	All India	0.33	100

In India, the disease scenario is changing quite rapidly and has been a constant challenge to the breeders and crop protection researchers for deployment of suitable management strategies. *Alternaria* leaf spot was a major threat till the Eighties, followed by sunflower necrosis disease caused by tobacco streak virus since its first appearance in 1997 (Basappa and Santhalakmi Prasad, 2005). In India, the occurrence of powdery mildew was first reported on Mexican sunflower in the year 2008 (Baiswar et al., 2009) and subsequently on cultivated sunflower in 2009. In the first two years of its occurrence, it was observed only during the spring season and at flowering and post flowering stages, but subsequently, it has

become serious during all crop growing seasons and often infecting the crop at vegetative stage itself. During the past decade, powdery mildew caused by *Golovinomyces orontii* has become a severe problem and has spread rapidly to all sunflower cultivation regions of the country (Reddy et al., 2013; Sujatha et al., 2015). Powdery mildews have world-wide distribution, but greater intensity on sunflower is reported in the tropical regions (Zimmer and Hoes, 1978; Diaz-Franco, 1983; Gulya et al., 1997; Anyanga and Biruma, 2010). The disease is reported to cause yield losses up to 13% in Mexico (Diaz-Franco, 1980), 25% in USA between 1983 and 1989 (Gulya et al., 1991) and 30-74% in India depending on the disease severity (Dinesh et al., 2010; Sujatha et al., 2015; Madhusudhan et al., 2017).

2.2. Powdery mildew

2.2.1. Powdery mildew

2.2.1.1 Life cycle of powdery mildew and dissemination

The conidia of powdery mildew form cell wall and cells contain nuclei, vacuoles, woronin bodies like other filamentous ascomycetes. They exhibit pleomorphism. The life cycle of powdery mildew includes both telomorph (sexual state) and anamorph (asexual state). Sometimes, either one of them is lacking. The life cycle of powdery mildew can be described based on three aspects: infection, reproduction and perennation.

2.2.1.2. Infection: Conidium germination is initiated when an ascospore or conidium lands on a suitable host. Soon after its germination, it forms a germ tube that elongates to form a hypha with appresoria, penetration peg and haustoria. The penetration peg penetrates into host cell and extened to form haustorium, which helps in maintaining the parasite relationship with the host cell.

2.2.1.3. Asexual reproduction: Immediately after infection of powdery mildew on the host, hyphae elongate and branch repeatedly, forming circular colonies. The hyphae give rise to the development of reproductive structures (conidiophores and/or chasmothecia). Within few days of infection, the candle production begins. The conidiophores originate from vegetative cells and most of them are perpendicular to the surface of the host. The conidial production is basauxic (Moriura et al., 2006; Oichi et al., 2006). Generally, powdery mildew conidiophores are unbranched (Fig. 2.1). The conidia are single cells and range in shape from ovoid to cylindrical or lanceolate.

The release of conidiophores occur either by mechanical force (Plumb and Turner, 1972), wind (Grove, 1998), convection currents (Frinking and Scholte, 1983), electrostatic

charges (Adams et al., 1986; Leach and Apple, 1984), and leaf shaking or fluttering. The dissemination of spores follows a diurnal pattern and negatively correlates with high humidity (Grove, 1998; Hammett and Manners, 1971; Sutton and Jones, 1979). Conidia are spread either singly or in short chains (Schnathorst, 1959).

2.2.1.4. Sexual reproduction: Chasmothecium is the term used for powdery mildew ascomata that distinguish it from other ascomycetes. Chasmothecium is initially light colored which turns into dark brown to black upon maturation (Braun et al., 2002). The sexual reproduction in powdery mildew begins with the production of gametangia. The plasmogamy was established with connection between gynogamocystes (female) and androgamocytes (male). The nucleus moves from male gamocytes to female gamocytes and becomes a binucleate or dikaryon, and the hyphae produced by monokaryotic cells forms the base of the ascogonium, which grow to envelop it and forms the developing peridium. Dikaryotic cells result in development of asci. Ascospores (asci) are single-celled ranging from globose to ovoid in shape. In general, the dissemination of ascospores happens in rain events (Grove, 1991; Jarvis, 2002).

2.2.1.5. Perennation: Perennation is the process of survival of powdery mildew during unavailability of susceptible host (Braun, 1987). Perennation in powdery mildew occurs in three modes *viz.* First mode is by chasmothecia adaptation and serves as resistant structure during winter and dry summer. Second mode is by the process called bud perennation, where it forms a dominant bud. The third mode is mycelia adaptation to hot or winter.

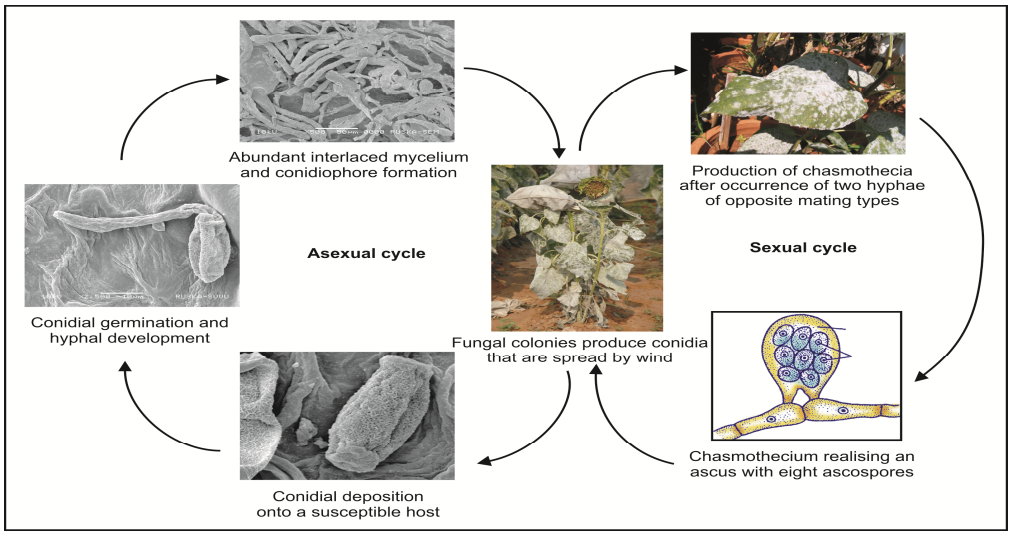

Fig. 2.1 Life cycle of powdery mildew in sunflower (***source of chasmothecium image*** *: http://www.biologydiscussion.com/essay/essay-on-fungi-biology/21760*)

2.2.2. Identification of powdery mildew genus, that causes disease in India by internal transcribed spacer (ITS) primers

Powdery mildew is cosmopolitan in nature. The order Erysiphales contains one family, the Erysiphaceae and 19 genera (Kirk et al., 2008). Identification of powdery mildews on members of the Asteraceae is not simple. It is reported that at least three genera of powdery mildews *viz., G. orontii* (formely *G. cichoracearum*), *Leveillula taurica* and *Podosphaera xanthii* are the causative agents of powdery mildew in sunflower (Saliman et al., 1982; Gulya et al., 1991; Fang 1973; Anonymous 1994; Yang et al., 1988). Before embarking on a large-scale screening program it is necessary to confirm the pathogen causing the disease. The molecular detection methods for identification of the pathogens based on ITS sequences were found to be superior over microscopic methods (Hirata and Takamatsu 1996; Takamatsu and Kano 2001; Chen et al., 2008). The set of ITS primers designed by Chen et al. (2008) were used in this study to confirm the genus that caused epidemics of powdery mildew on sunflower in Southern India.

2.2.3. Scoring scales for powdery mildew disease assessment

Among the economically important crops are wheat, barley, cucurbits, pea, tomato, chili, pepper, mungbean, grapes and mulberry. Disease assessment/scoring was done on a 0-9 scale in Wheat (Saari and Prescott 1978), in pea (Warkentin et al., 1996) in chili (Mayee et al., 1986), 0-4 scale in barley (Mains and Dietz 1930; Torp et al., 1978; Kolster et al., 1986) and cucrbits (Lebeda, 1984), 0-3 scale in tomato (Lebeda and Mieslerova, 2010), a 0-5 scale was used in pepper (Lefebvre et al., 2003) and in mungbean (Reddy et al., 1994) in grapes a 0-7 scale (Wang et al., 1998), in mulberry a 0-10 scale was used (Chattopadhyay et al., 2010).

Different disease scoring scales were used for estimation of powdery mildew disease incidence in sunflower *viz.,* a 0-3 scale (Saliman et al., 1982; Dedic et al., 2012), percentage of leaf area infected (Jan and Chandler 1985; Roja-Barros et al., 2004; 2005; Gulya et al., 1991) and a 0-5 scale (McCarter 1993; Dinesh et al., 2010). In the previous reports, disease evaluation was confined to wild *Helianthus* species and the material was scored as either resistant or susceptible since only 2 disease phenotypes were discernible. As the material used for screening sunflower germplasm for powdery mildew resistance in the present study was from diverse genetic backgrounds exhibiting significant variations in qualitative and quantitative characters, infection was observed on the whole plant or confined till the middle

leaves or only to the lower leaves. Hence, there is a need to develop a new scoring scale based on the level of infection on the bottom, middle and top leaves

2.2.4. Control of powdery mildew in sunflower

In general, fungicides are largely used for the management of powdery mildew disease in sunflower. Fungicides like bayleton, tilt, propiconazole, difenoconazole, carbendazin, hexaconazole macozeb and wettable sulphur have significantly reduced the powdery mildew incidence. The new chemicals like topas, clone, aerosol, cumulus and sufex gold were found to be superior in controlling powdery mildew disease (Dinesh, 2009; Akhileswari et al., 2012; Yasir et al., 2016; Madhusudhan et al., 2017). Since powdery mildew appears at flowering and post flowering stages at which time, spraying of fungicides is not done as it affects pollination, there is a need to focus on the resistance breeding programs through the utilization of resistance sources from cultivar germplasm to minimize the losses due to diseases.

2.2.5. Identification of resistance sources in sunflower wild species and cultivated germplasm against powdery mildew

In earlier studies, sources of resistance have been identified in wild sunflowers. In studies of Saliman et al. (1982), the wild species *H. atrorubens, H. californicus, H. ciliaris, H. debilis, H. decapetalus, H. laciniatus, H. laevigatus, H. microcephalus, H. resinosus, H. rigidus, H. simulans* and *H. smithii* showed resistance at field, the two annuals *H. bolanderi* and *H. praecox* ssp. *praecox* showed resistance under artificial inoculation assays while two annual species *H. debilis* Nutt. ssp *silvestris, H. divaricatus* and the perennial species *H. strumosus* showed resistance under both field and artificial inoculation conditions. Interspecific hybrids between *H. giganteus, H. hirsutus, H. divaricatus, H. salicifolius* and cultivated sunflower showed no symptoms of powdery mildew (Skoric 1984). In the annual wild species *H. debilis*, the resistance was governed by a partial dominant gene and the interspecific hybrid (PM1) between *H. debilis* and *H. annuus* was registered as a source of resistance to powdery mildew (Jan and Chandler 1985; 1988). Among the thirty six genotypes of Jerusalem artichoke (*H. tuberosus*), two genotypes showed resistance to powdery mildew (McCarter 1993). Three subspecies of *H. debilis* showed resistance to powdery mildew (Rojas-Barros et al., 2004) and these sources were different from that identified by Jan and Chandler (1985).

Transfer of powdery mildew resistance genes from *H. debilis* ssp. *debilis* and *H. argophyllus* into cultivated sunflower indicated that resistance in both cases is controlled by at

least two genes (Rojas-Barros et al., 2006). Five perennial wild species, *H. decapetalus, H. laevigatus, H. glaucophyllus*, *H. ciliaris, H. tuberosus* and one annual species *H. debilis* showed resistance to powdery mildew; resistance in *H. decapetalus* was controlled by a dominant gene where as the resistance in the other species was quantitative (Christov 2008). Resistance to this pathogen was also found in some accessions of *H. tuberosus, H. praecox, H. bolanderi* and *H. praecox* (Acimovic 1998). Screening of a total of 333 accessions of 23 perennial *Helianthus* species against powdery mildew under natural infection conditions showed resistance in 124 accessions representing 23 perennial species (Dedic et al., 2012). The studies till date report the sources of resistance from wild species while reports on resistance sources in cultivated germplasm are lacking. However, it is interesting to note that some of the diploid annuals that are easily crossable with cultivated sunflower (*H. debilis, H. praecox* and *H. bolanderi*) confer resistance to the pathogen (Saliman et al., 1982; Jan and Chandler 1985; 1988; Rojas-Barros et al. 2004; 2006; Christov 2008). Screening of wild *Helianthus* species and cultivated sunflower accessions resulted in identification of sources of resistance to powdery mildew in both wild and cultivated sunflower (Saliman et al., 1982; Jan and Chandler, 1985; 1988; McCarter, 1993; Rojas-Barros et al., 2004; 2006; Dedic et al., 2012; Reddy et al., 2013; Kulkarni et al., 2015).

2.3. Histopathology of powdery mildews

Powdery mildews are considered to be the best models for basic research on obligate parasite and plant interactions, developmental morphology, cytology, and molecular biology. However, recent research had shown the complexity in powdery mildews. There is great difficulty in identifying and characterizing of powdery mildews due to their diversity and their biology is more complex than generally realized (Yarwood, 1978; Braun et al., 2002). The detailed characteristics of powdery mildews are outlined below.

2.3.1. Identification and classification of powdery mildews on other hosts than sunflower: Role of light and electron microscopy

The causal agents of powdery mildew are obligate, biotropic parasites of the phylum Ascomycota of kingdom Fungi (Braun et al., 2002). Powdery mildew pathogen infects leaves, stems, flowers, and fruits of about 10,000 species of angiosperms (Braun et al., 2002). Among the economically important plant species that are infected by powdery mildew are cereals (e.g. wheat and barley), cucurbits (e.g. sqaush), Solanaceae members (e.g. tomato), grapevine and ornamental plants (e.g. roses), (Adam and Somerville, 1996). Unlike other

fungi, powdery mildew grows epiphytical on the surface of the host. Hyphae are produced on both upper and lower leaf surfaces of the host.

The fine structure of conidia and hyphae of *Erysiphe graminis hordei* were investigated (Akai et al., 1968). The electron micrographs showed that both the conidia and hyphae of *E. graminis* are smooth cell walled with a thick outer layer and an electron-transparent inner layer, with, a specific lamellar structure, which was present in conidia and absent in the hyphae. A total of 36 *Ersiphae spp*. were studied for their modes of branching of the appressoria on conidial germ tube (Cook et al., 2011). Light and scanning micrographs revealed that, a combination studies of development of appressoria on germ tube and germ tube length is very useful in identification and differentiating the different species of *Ersiphe*. Light and scanning electron microscopy were used in investigating the conidial germination, infection structure formation and early colony development of powdery mildew on poinsettia (Celio and Hausbeck, 1998). The emphasis of the study was at 20 ^{0}C, and the appressorium formation was observed within 6 hours of post inoculation. A colinearity exists between the percentage of germinated conidia with appressoria, secondary germ tube and time. But at 30 ^{0}C, conidial germination and development of secondary germ tubes and hausturium were reduced. Cook and Braun (2009) had given a detailed catalogue of the newly categorized conidial germination patterns and a dichotomous key for the identification of powdery mildew anamorphs.

The combined use of classical morphological (light microscopy) and SEM analysis had successfully compared the morphological characteristics of *Oidium* tomato pathogens from all over the world (Kiss et al., 2001). The conidial germination and infection process of endoparasitic powdery mildew fungus, *Leveillula taurica* on green pepper leaves was studied (Kunoh et al., 1979). The successful monitoring of conidiophores of *Blumeria graminis* on barley leaves was done by using a digital microscopic technique (Moriura et al., 2006). The study determines the time interval between conidial releases and to count the total conidia that seeded from individual living conidiophores during life time. The formation of conidial pseudochains by *Oidium neolycopersici* (tomato powdery mildew) was determined by using digital microscope (Oichi et al., 2006). The study concludes that *O. neolycopersici* continuously develops conidia at the conidiophores apex and conidia in pseudo chains are wind dispersed during strong wind condition.

A combination of light microscopy, SEM and transmission electron microscopy was used in studying morphology, wall structure, and initial adhesion of conidia of the powdery mildew fungus *Uncinuliella australiana* that infect crape-myrtle (Mims et al., 1995). The observed conidia were ellipsoid cylindrical in shape, transparent and thin walled. Conidia have minute longitudinal surface ridges that extended along most of the length of each conidium. An adhesion pad was observed between the underside of a conidium and the membrane surface appeared to be the initial step in the process of conidial adhesion. The conidial chains and conidial development of powdery mildew fungus *Erysiphe graminis* that infects wheat was studied using scanning electron microscopy (Plumb and Turner, 1972). The study described the development of spiny conidium and infection process on wheat leaves. A comparative study on spore germination, infection structure formation, and colony development of *Ersiphe pulchra* on glass slides and leaf disks of a susceptible dogwood was described (Windham and Trigiono, 2005). Cook et al. (2006) had first reported *Neoerysiphe galeopsidis* on *Acanthus spinosus* in the UK by using SEM. Which elucidated the structural features of *N. galeopsisis*. Three species of powdery mildew, *Erysiphe elevata, E. catalpse,* and *Neoerysiphe galeopsidis* were identified on Catalpa species using morphological features supported by molecular data (Cook et al., 2006).

2.3.2. Factors affecting primary infection of powdery mildew on host

There were several important factors that control the infection process of powdery mildews. The first factor is the type of epidermal cell on leaf abaxial or adaxial surface of the leaf, which has an effect on infection of powdery mildew (Koga et al., 1990). The infection rate in short cells ranged from 73 to 79%, compared to 15-16% in long cells. The fungus *Blumeria graminis* (causes barley powdery mildew) was well adapted in short cells than long cells. The infection rates in newly emerging leaves were more (60-70%), compared with fully expanded leaves (10%) (Lin and Edward, 1974). The decrease in the infection rate of pathogen in barley with age was reported (Nelson et al., 1990). But in dicotyledonous crops such as cucumber, tobacco and tomato, there was an increase in powdery mildew colony development with age (Jarvis et al., 2002). Rate of powdery mildew infection was also controlled by spore density and the infection rate of powdery mildew decreased with increase in spore density (Carver and Inger 1989).

2.3.3. Germination of powdery mildew conidia

Conidial germination and infection process were studied in detail in *Blumeria graminis* and *Erysiphe pisi*. Conidial germination occurs only after separation from conidiophores, and they do not germinate within the mother colony (Carver and Ingenson, 1987; Kunoh et al., 1992; Carver et al., 1999). In *B. graminis,* the ungerminated conidium releases a proteoneous conidial extracellular material (ECM) (Kunoh et al., 1992). The ungerminated conidia release liquid exudates upon contact with the substratum, which results in changes in the morphological characteristics of conidia. It results in changes in the morphological characteristics of conidia; where by the surface projections on the conidial surface develops a rounded rather than spine like appearance (Kunoh et al., 1988).

The exudates contain a cutinase (Pascholati et al., 1992) and hydrolytic enzymes (Nielsen et al., 2000) and release esterase (Nicholson and Kunoh 1995). These exudates degrade the surface cuticle of barley (Kunoh et al., 1990; Nicholson et al., 1993). The release of conidial exudates would decrease the relative hydrophobicity of conidia which builds a strong association with host (Nicholson et al., 1993). However, it is very difficult to visualize the conidial ECM on host by using SEM (Carver et al., 1999).

2.3.4. Emergence of germ tube in powdery mildew

Light microscopy and SEM studies of powdery mildews show that germ tubes emerge most frequently from the conidia close to the site of its contact with the host leaf (Green et al., 2002). In *B. graminis* the first formed germ tubes of more than 80% of conidia emerged from the area of conidial wall facing the host (Green et al., 2002). The first formed germ tube made contact with the plant surface and functioned as a primary germ tube (PGT). And the second germ tube also makes contact with the plant surface, elongated and differentiated as an appressorium. Nielsen et al. (2000) argued that signaling for the directional emergence of germ tubes may involve the uptake of external factors, including anionic molecules from the host surface via conidial extra cellular material (ECM).

2.3.5. Role of primary germ tube (PGT)

The role of primary germ tube (PGT) was well studied in the barley powdery mildew pathogen (*B. graminis*). The first germ tube that gets contact by the host is likely to become the functional PGT. The primary function of PGT is to form a rapid attachment between fungal germ line and host. The ungerminated conidia can be easily washed out from the host (Carver and Bushnell, 1983). The second function of PGT is to gain access to host water

(Carver and Bushnell, 1983). PGT produces a short peg, which penetrates the host surface to an unknown depth (Takamatsu et al., 1978). This provides a route by which the fungus may gain access to host water and other host components. The third important function of PGT is to recognize characteristics of the contact surface (Carver and Ingerson, 1987). The purpose of this recognition is to engage intracellular signaling that results in elongation of the next formed germ tube, a prerequisite to appressorium formation. Cellulose degrading enzymes were produced at the tips of PGT (Suzuki et al., 1998; Pryce-Jones et al., 1999).

2.3.6. Appressorial germ tube formation and differentiation of the appressorium

The elongated germ tube in either *B. graminis* or *E. pisi* would remain as an undifferentiated, hypha-like structure unless it makes contact with and responds to inductive characteristics of the underlying substratum (Carver and Ingerson, 1987; Carver et al., 1999). Substratum hydrophobicity does appear to stimulate appressorial lobe differentiation by *E. pisi* (Carver et al., 1999). When the elongated germ tube of *B. graminis* or *E. pisi* makes contact with a leaf surface or other suitable substratum, the amorphous extracellular material is secreted beneath the elongating tube (Carver et al., 1999). The extracellular material may act as a matrix for the fungal enzymes involved in host surface degradation (Carver et al., 1999).

This extracellular material contains enzymes, which are involved in adhesion of germ tubes, breakdown of the cutin and cellulose that are taken up by the fungus leading to intracellular signaling for differentiation of appressorial lobes. Apart from extracellular material, cAMP concentration and its dependent protein kinase (PKA) play a key role in signal transduction and intracellular cascades which drive morphogenesis in powdery mildews (Kinane et al., 2000).

2.3.7. Penetration of the host cell

Penetration of the outer layer of the host surface occurs by cutin degradation by cutinase activity (Keon et al., 1987; Howard et al., 1991). Furthermore, the penetration is assisted by hydrolytic enzymes (Fric and Wolf, 1994). In *B. graminis,* conidial and germ tube pectinase gene expression has been described (Suzuki et al., 1998). In case of *M. grisea,* adhesion of the appressorium and the production of a pore ring of extra cellular fungal material was thought to seal the interface between the fungus and the substratum (Howard et al., 1991). Extracellular material can be seen surrounding the penetration sites in the leaf surface and this may be equated to the pore ring, scaling the penetration site and allowing build up of the turgor.

In case of *B. graminis* a combination of enzymatic and mechanical forces act together in facilitating the entry of the pathogen into the host (Edward and Allen 1970; Zeyen et al., 2002). Thus penetration can be categorized into two stages, the first stage involving enzymatic hydrolysis of the epidermal cell wall and the second one, which is a force full puncture of the papilla. The mechanical force was created with high turgor pressure by appressoria. This would involve the production of high concentrations of an osmolyte by the hydrolysis of conidial storage sugars (De Jong et al., 1997).

2.3.8. Differentiation and development of the haustorial complex

Immediately after penetration of the host cell wall and papilla, the tips of the fine hyphal penetration peg enters the epidermal cell and grows to form a specialized absorption structure termed as haustorium (Green et al., 2002). The hyphal penetration peg that grows through the papilla constitutes the haustorial neck, which is a small tube connecting the haustorium to the surface mycelium. Morphological features of hyphae differ from species to species, but most of them are oblong or pear shaped (Bushnell, 1972; Heath and Skatamera, 1997). Sometimes haustoria vary in size and shape with age (Gil and Gay, 1977).

In order to increase the surface area of haustorium, projections known as haustorial lobes branch out from the haustorial body (Manners and Gay 1983). The haustorium was comprised of haustorial cytoplasm, haustorial plasma membrane, and haustorial wall. During the formation of haustoria within epidermal cells of the host plant, they become enclosed by an invagination of the host plasma membrane called the extra haustorial membrane (EHM). The EHM was separated from the haustorial walls by the extra haustorial matrix (EHMAT). The collective term for combination of haustorium. EHMAT and EHM were called as houstrial complex (HC) (Gil and Gay, 1977).

2.3.9. The development of the haustorial complex (HC)

The formation and growth of powdery mildew fungus was mostly similar to that of general fungal mechanisms. The only exception in powdery mildews is that the haustorial complex was a determinate structure that reaches finite size and remains within the epidermal cell that it initially infects. Though the fungus gets benefited by association with the host plant, the formation of a functional HC requires some input from the plant. The EHM expands during the development of the HC and it requires membrane synthesis and was probably derived from the plant (Green et al., 2002). Endoplasmic reticulum (ER) of the plant had a

significant role in signaling between the plant and fungus during HC formation (Leckie et al., 1995).

2.3.10. The function of HC

Haustorium complex plays a key role in nutrient uptake by powdery mildew from the host plant during plant-powdery mildew interactions (Manners and Gay, 1982). In resistant host plant, haustorial growth is commonly stunted or arrested prematurely, thus preventing biotrophy and the development of secondary hyphae and conidiophores (Dickey and Leuy, 1979).

2.3.11. Quantification of powdery mildew colony development

After a successful penetration into the host, the primary haustorial complex is developed. Subsequently, the secondary hyphae are formed, which spread epiphytically to the neighboring host cell, and thus develop into colonies and produce characteristic white powdery mildew pustules (Micali et al., 2008). The quantification of powdery mildew infection and comparative infection levels between resistant and susceptible hosts can be studied by three major methods, i.e. macroscopic categorization, microscopic-based penetration count and hyphal growth and conidiophores count (Reuber et al., 1998; Vogel et al., 2000; Consonni et al., 2006).

Macroscopic categorization of resistant and susceptible phenotypes involves disease scoring by naked eye at late stages of pathogenesis (Reuber et al., 1998; Vogel et al., 2000; Seiffert et al., 2005; Gollner et al., 2008; Consonni et al., 2010; Baum et al., 2011). The categorization of resistant and susceptible hosts is based on the severity of disease symptoms (Reuber et al., 1998; Humphry et al., 2010). The other methods involve quantitative estimation of host cell entry by penetration counts (Consonni et al., 2006; 2010) or by counting the number of conidiophores for characterizing the powdery mildew resistant mutants (Consonni et al., 2006; Reuber et al., 1998; Vogel and Somerville, 2000). The recent method developed for quantification is by employing qPCR (Webing and Panstruga, 2012).

2.4. Biochemical response of the plants to powdery mildews

Plant deploys various mechanisms to combat the pathogen attack. The determinants of resistance have been studied using genomic, transcriptomic, biochemical and proteomic techniques (Michelmore, 2000; Rampitsch and Bykova, 2012; Lodha et al., 2013; Thorpe et al., 2014). One of the important responses from a host to its biotrophic fungal counterpart is the hypersensitive reaction (HR). A pathogen attack triggers the generation of reactive

oxygen intermediates (ROIs) by a variety of enzyme systems (Bolwell et al., 2002; Babitha et al., 2004; Deepak et al., 2006; Zimmermann et al., 2006; Bindschedler et al., 2006; Cona et al., 2006; Carter et al., 2007; Amirsadeghi et al., 2007). ROIs are involved in various signalling pathways for defense mechanisms such as triggering of the HR, accumulation of phytoalexins and a number of other defense response genes. The concentrations above the threshold level of ROIs has the potential to cause oxidative damage by reacting with biomolecules, whereas they act as powerful signalling molecules that are involved in the regulation of plant growth and development as well as priming acclimatizing responses to the stress stimuli, when they are below the threshold levels (Bowler and Fluhr, 2000; Foyer and Noctor, 2009; Yadav et al., 2016). The synergy between the antioxidant enzymes and redox metabolites modulates the steady-state levels of superoxide radicals and hydrogen peroxide.

2.4.1. Reactive Oxygen Intermediates

Reactive oxygen intermediates (ROIs) are derived from molecular oxygen. They are particularly reduced forms of atmospheric oxygen and are a result of step wise excitation of O_2 to form singlet oxygen (O_2^-) or from the transfer of one, two or three electrons to O_2 to form, respectively, a super oxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-). These ROIs are able to bring unrestricted oxidation of various cellular components and can lead to the oxidative damage of the cell (Asada, 1999; Dat et al., 2000). In plants, the levels of ROIs would be low under normal conditions, but the production of high level ROIs is induced at either ambitious or biotic stress conditions. This stress condition triggers the active production of ROIs (Hammond-Kosack and Jones, 1996; Cazale et al., 1999; Pei et al., 2000). Higher concentration of ROIs have the potential to cause oxidative damage by reacting with biomolecules, whereas, relatively low levels act as powerful signaling molecules involved in the regulation of plant growth and development as well as priming acclimatory responses to the stress stimuli (Foyer and Noctor, 2009), and cross-talk (Bowler and Fluhr, 2000), where an encounter with one stress leads to greater response to a second similar exposure, or to other stresses. The mode of action of ROIs as signaling molecules or causing oxidative damage to the tissues depends on the delicate equilibrium between ROIs production and their scavenging (Yadav et al., 2016). ROIs act as a cellular indication of stress and as secondary messengers involved in the stress-response signal transduction pathway (Mittler, 2002).

ROI family consists of the superoxide radical anion (O_2^-), the hydroperoxyl radical (HO_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO). The free radicals O_2^-, HO, HO_2, have short life span, whereas H_2O_2 is relatively stable and can pass through the membrane (Hückelhoven and Kogel, 2003). Among the four free radicals, the best studied free radicals are O_2^- and H_2O_2 as they are comparatively more stable than the other two short lifespan free radicals (Baker and Orlandi, 1995; Hammond-Kosack and Jones, 1996; Grant and Loake, 2000). The level of ROIs accumulation in host plant regulates the induction of plant defense reaction against viral, bacterial, and fungal pathogens by inducing the hypersensitive reaction (HR), defense gene expression, and cell wall strengthening via cross linking reaction of phenyl propane and proteins (Thordal Christensen et al., 1997; Lamb and Dixon, 1997; Grant and Loake, 2000; Shetty et al., 2008).

In compatible host, the pathogens are able to provoke disease, whereas in an incompatible host, defense mechanisms block the penetration of the pathogen and block their spread. The hypersensitive response (HR) in incompatible host results in localized cell death and leads to impaired pathogen spread. The synergy between enzymes and redox metabolites, modulate the steady-state levels of superoxide radicals and hydrogen peroxide.

2.4.2. Generation of ROIs during biotic stress

In plants, ROIs are produced continuously as a byproduct of various metabolic pathways (Foyer and Harbinson, 1994). Under normal physiological conditions, the steady state of these molecules was maintained by scavenging of ROIs in the cell by different antioxidative defense components (Alscher et al., 1997). Environmental conditions play a key role in maintaining the equilibrium between the production and scavenging of ROIs in plants. Plants also generate ROIs by activating various oxidases and peroxidases in response to environmental conditions (Allan and Fluhr, 1997; Bolwell et al., 1998; Doke, 1985; Schopfer et al., 2001). Depending upon the stress conditions, there are several potential sources of ROIs in plants (Lamb and Dixon, 1997; Bolwell et al., 2002).

A pathogen attack triggers the generation of ROIs by a variety of enzyme systems, i.e. the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase (SOD), oxalate oxidases, lipoxygenases, and amine oxidases (Cona et al., 2006; Deepak et al., 2006; Zimmermann et al., 2006; Bindschedler et al., 2006; Carter et al., 2007). During pathogenesis, a pressure was developed on the ROI producing organelles and results in production of ROIs (Apel and Hirt 2004; Amirsadeghi et al., 2007) (Fig. 2.2).

In plants ROIs generation occurs in a biphasic manner; when an avirulent pathogen attack, the first phase was a transient phase with small levels of ROIs accumulation followed by a continuous phase of much higher levels, that correlates with oxidative burst and disease resistance (Lamb and Dixon 1997; Torres et al., 2006). In some cases it may be of three phases (Huckelhoven and Kogel, 2003; Shetty et al., 2003). Induction of either two phase ROIs accumulation or three phase ROIs accumulation depends on the pathogen and genotype of the host.

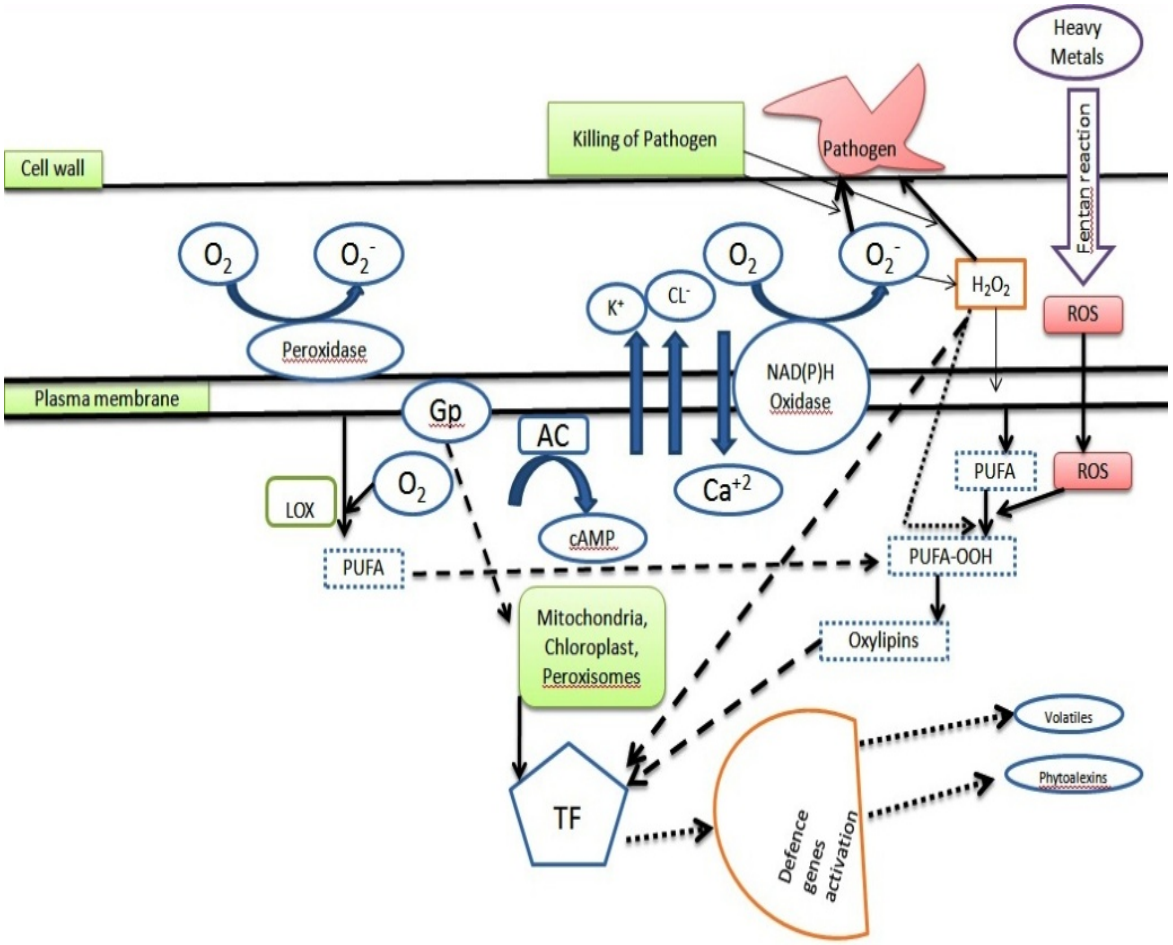

Fig. 2.2 Model for production of ROS in response to various stresses and their role in signal transduction- *source* Yadav et al., 2016

2.4.3. Roles of ROIs in host-pathogen interaction

Activation of signal transduction results in high levels of ROIs accumulation and activation of defense genes coding for PR-proteins, phytoalexin generating enzymes, enzymes involved in oxidative stress protection, lignifications and other defense responses (Alvarez et al., 1998; Apel and Hirt, 2004; Lamb and Dixon, 1997). The HR response was triggered only by balanced production of NO and ROS (Delledonne et al., 2001). The HR is a quick host response resulting in a host, infected by a pathogen (Lam et al., 2004). The cells, which are infected by pathogen die shortly after penetration, and often the surrounding cells also (Greenberg, 1997; Van Breusegem and Dat, 2006). In case of biotropic pathogens, HR is an effective method of restriction of the pathogen by host, since, with the death of host cells, the nutrient supply is depleted (Mellersh et al., 2002; Greenberg and Yao, 2004).

The role of ROIs in HR has been studied by different approaches, including infiltration of antioxidants (Li et al., 2006) and ROIs inhibitions or scavengers (Sasabe et al., 2000; Li et al., 2006). Plants mutated in their ability to accumulate ROIs or express antioxidants (like SOD, catalase and ascorbate peroxidase) and activation of HR were studied (Dat et al., 2003; Lorrain et al., 2003; Mateo et al., 2004; Montillet et al., 2005; Van Breusegem and Dat, 2006). Several studies have shown a correlation between the accumulation of ROIs (H_2O_2, O_2, O_2^-), NO and HR (Dat et al., 2003; Montillet et al., 2005). On the other side, there are reports on the lack of correlation (Dorey et al., 1999; Repka, 2002; Torres et al., 2006). Thus, the exact role and mechanism of ROIs in elicitation of the HR remains somewhat unclear (Shetty et al., 2008). There could be other higher levels of regulation of balanced production of H_2O_2 and NO in HR response (Delledonne et al., 2001). Thus the role ROIs for activation and executing the HR is complicated and influenced by many factors.

There are studies suggesting the role of ROIs on both bitropic pathogen growth inhibition (Thordal-Christensen et al., 1997; Mellersh et al., 2002) and successful pathogenesis (Link et al., 2005; Voegele et al., 2005; Ferreira et al., 2007). In case of necrotropic pathogens, ROIs such as H_2O_2 would benefit infection process (Govrin and Levine 2000). In hemibiotropic pathogens, a correlation between pathogen growth at late stages of life cycle and large quantities of H_2O_2 has also been reported in such host-pathogen systems (Able, 2003; Shetty et al., 2003). It is observed that in wheat infected with hemibiotropic pathogen *S. tritici*, H_2O_2 accumulation occurred as a defense response only in

an incompatible interaction of biotropic phase. On the other hand, in a compatible interaction, large amounts of H_2O_2 accumulation after extensive tissue colonization was witnessed just before the appearance of symptoms and sporulation of the pathogen (Shetty et al., 2007). This complicated role of ROIs in host-pathogen interaction reveals that the role of ROIs will be high divergent and definite for a specific type or even species of pathogens (Shetty et al., 2008).

2.4.4. Enzymatic ROIs- scavenging mechanisms in plants

The major ROIs scavenging mechanisms that exist in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Asada and Takahashi, 1987; Bowler et al., 1992; Wilekens et al., 1997). The balance in SOD, APX and catalase was crucial for maintaining the superoxide radicals and hydrogen peroxide (Bowler et al., 1991). The CAT and APX belong to two different classes of H_2O_2 scavenging enzymes. APX regulates the fine modulation for signaling, whereas CAT removes the excessive ROIs during stress (Mittler, 2002).

The principle mode of ROIs in plant includes four major pathways (Mittler, 2002; Apel 2004) (Table 2.3). The first pathway of scavenging ROIs includes SOD, found in all cellular compartments, the water-water cycle in chloroplast The second mode of reducing ROIs includes the ascorbate-gluthione cycle in chroloplasts, cytosol, apoplast, mitochondria and peroxisomes. The other two pathways include glutathione peroxidase (GPX) and CAT in peroxisomes. In addition to the enzymatic ROIs scavenging mechanism, non-enzymatic ROIs antioxidants such as ascorbic acid and glutathione are also playing a crucial role for plant defense against oxidative stress.

Table 2.3 Localization of reactive oxygen species and the mechanism of scavenging in plants

Scavenging system	Reaction catalysed	Localization
SOD	$O_2^{\cdot-} + O_2^{\cdot-} + 2H+ \rightarrow 2H_2O_2 + O_2$	Chl, Cyt, Mit, Per, Apo
APX	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	Chl, Cyt, Mit, Per, Apo
CAT	$2\ H_2O_2 \rightarrow O_2 + 2H_2O$	Per
GR	$GSSG + NADPH \rightarrow 2GSH + NADP+$	Cyt, Chl, and Mit
POX	$H_2O_2 + DHA \rightarrow 2H_2O + GSSG$	CW, Cyt, Vac
MDHAR	$2MDHA + NADH \rightarrow 2AA + NAD+$	Chl, Cyt, Mit
DHAR	$DHA + 2GSH \rightarrow AA + GSSG$	Chl, Cyt, Mit
AA	Detoxifies H_2O_2, Substrate for APX.	Chl, Cyt, Mit, Per, Apo
GSH	Substrate for various POXs, GSTs and GR. Detoxified H_2O_2, other hydro peroxidases and toxic compounds	Chl, Cyt, Mit, Per, Apo

Scavenging system	Reaction catalysed	Localization
α -Tocopherol	Protects membrane lipids from peroxidation, detoxifies lipid peroxides, and quenching $^1O_2$	Membranes
Carotenoids	Quench $^1O_2$. Photosystem assembly, key components of the light harvesting complex, precursors for abscisic acid (ABA)	Chl
Phenolics	Can directly scavenge H_2O_2 and $OH^·$	Vac

Chl-chloroplast, Cyt-cytosol, Mit-mitochonderia, Per-peroxisomes, Apo- apoplast, CW-cell wall, Vac-Vacuole

Source-Yadav et al., 2016

2.4.5. Peroxides (POX; EC 1.11.1.7)

Generation of ROIs during the oxidative burst involves two major mechanisms i.e. plasma membrane NADPH oxidases or cell wall peroxidases (Grant et al., 2000; Torres et al., 2006). Plant peroxidases catalyse the reduction of H_2O_2 by transfering electrons to different donor molecules such as lignin precursors, phenolic compounds, auxins or secondary metabolites (Hiraga et al., 2001). Peroxidases play a key role in various physiological processes, such as lignin and suberin formation, cross linking of cell wall components, auxin metabolism, and defense against pathogens. Peroxidases are activated during plant-pathogen interaction. Subsequent activation of peroxidases leads to the onset of oxidative burst and cell wall cross-linking activity, thus creating an adverse environment to the pathogen. Peroxidases carry out three important mechanisms in plants, *i.e.* ROS generation and regulation, H_2O_2 level regulation, and oxidation of various substrates. The existence of the high number of isoforms further allows a fine balance between antagonistic peroxidase functions such as cell wall cross-linking and loosening (Passardi et al., 2004). Several studies showed that peroxidase up-regulation is generally transient. Often, they were strongly induced at the beginning, which slowly decrease with time (Blee et al., 2001; Passardi et al., 2004).

During bacterial blight attack on cotton, the total peroxidase activity is high in the infected and localization mainly occurs at the apoplast and close to the bacterial infection site (Martinez et al., 1998). In rice upon infection with rice blight, strong upregulation of peroxidase isoform was detected at xylem parenchyma. This peroxidase was then secreted to the xylem vessels, resulting in secondary wall thickening and reducing the access of the pathogen to the contact point to the host cell (Hilaire et al., 2001). A strong increase in H_2O_2

was reported in lettuce upon challenge from bacterial inoculation (Bestwick et al., 1998). A large increase in peroxidase isozyme was correlated with resistance in wheat to stem rust disease (Seevers et al., 1971). Resistance to downy mildew disease in pearl millet was associated with an early increase in the peroxidase activity (ShivaKumar et al., 2003). Transgenic Arabidopsis plants with peroxidase gene silenced were impaired in oxidative burst and were more susceptible to both fungus and bacterial pathogen (Bindschedler et al., 2006). Peroxidase participates in the deposition of phenolic compounds in papillae and contributes to general, nonspecific defense response in barley seedlings infected with powdery mildew (Scott-Craig et al., 1995). Enhancement of intracellular and extracellular peroxidases is observed in barley attacked with powdery mildew fungi (Kerby and Somerville, 1989; Scott-Craig et al., 1995). Transient expression of vacuolar peroxidases enhances the susceptibility of epidermal barley to powdery mildew (Kristensen et al., 1999).

2.4.6. Superoxide dismutase (SOD; EC 1:15:1:1)

Superoxide dismutase plays a key role in plant defense mechanisms against biotic and abiotic stress. Free radicals generated during stress conditions are most reactive species that cause lipid peroxidation, the denaturation of proteins and mutation of DNA (Bowler et al., 1992). Suitable protective mechanisms are evolved to minimize the deleterious reactions. The elimination of ROS involves both enzymatic and non-enzymatic mechanisms. Superoxide dismutase react with superoxide radicals at almost diffusion limited rates to produce hydrogen peroxide (McCord and Fridovic, 1969).

$2O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$

The concentration of O_2^- and H_2O_2 is determined by SOD, because of which SOD plays a central role in the defense mechanism. Based on metal cofactors, SOD is broadly classified in to three types, *i.e.* Cupper/Zinc (Cu/ZnSOD), manganese (Mn SOD) and Iron (Fe SOD). In general, plants contain a mitochondrial matrix localized MnSOD and a cytosolic Cu/Zn SOD, with FeSOD and Cu/Zn SOD present in the chloroplast stroma. The isozyme numbers of each type of SOD differ greatly from plant and plant. Since SOD forms in H_2O_2 as a byproduct, it is immediately linked with Halliwell-Asada pathway of reducing oxidative stress. Thus SOD is cross linked with other enzymes and antioxidants in what is likely, a highly optimized balance that reduces the risk of free radical formation. The enzymes involved in free radical scavenging play an important role in determining the

consequences of plant-pathogen interaction and their presence at a subcellular location is very important (Bowler et al., 1992).

A resistant tobacco variety with elevated levels of SOD, but not catalase or peroxidase was resistant to cercospora, a fungal phytotoxin. Increased levels of Catalase and SOD activities in the roots of chickpea against *Fusarium oxysporum* were associated with enhanced resistance (Garcia-Limones et al., 2002). SOD activity was increased by 2-3 folds in resistant pearl millets seedlings upon inoculation with *Sclerospora graminicola* (Babitha et al., 2002). Upon foliar application of riboflavin and methionine (oxidative stress inducers) on cucumber, they develop resistance to powdery mildew by increasing their SOD and POD isozyme concentration (Nam Jun Kang, 2009). Potato virus V (PVV) induces higher levels of peroxidases, superoxide dismutase and catalase in tobacco, which in turns protect the tobacco from powdery mildew (Bunaurio and Montalbin, 1993). Many reports have been published on transgenic plants having enhanced levels of SOD exhibiting enhanced protection from oxidative stress (Van Camp et al., 1990; Gupta et al., 1993; Mc Kersie et al., 1996; Tertivanidis et al., 2004). Transcripts of sunflower antioxidant scavengers of the SOD and GPX families are differentially expressed in response to downy mildew and treatment with stress signaling factors. Thus antioxidant enzymes GPX and SOD are likely to play an important and different function in stress response (Herbette, 2003).

2.4.7. Ascorbate peroxidase (APX; EC 1.11.1.11)

Hydrogen peroxide is one of the molecular species of reactive oxygen species. If it was not scavenged immediately, it has the potential to produce highly reactive hydroxyl radicals by the transition metal catalyzed Haber-Weiss reaction. Thus, the generated hydroxyl radicals oxidize almost all the cell components at a diffusion controlled rate and cause cellular damages (Asada and Takahashi, 1987). Ascorbate-glutathione cycle is one main hydrogen peroxide-detoxification system in plants and APX is a key enzyme in the cycle (Asada, 1999; Noctor and Foyes 1998; Shigeoka et al., 2002; Mittler et al., 2004). APX utilizes Ascorbate as a specific electron donor to reduce H_2O_2 to water with the concomitant generation of mono dehydro ascorbate (MDASA) (Shigeoka et al., 2002). Studies on the response of APX expression against both abiotic and biotic stresses indicate the crucial role played by APX in controlling the H_2O_2 concentration in intracellular signaling.

The higher levels of APX in powdery mildew resistant wheat as compared to the susceptible line contributed significantly to eliminating ROIs (Chen et al., 2006).

Upregulation of APX gene was reported in barley leaves after inoculation with powdery mildew. In tobacco, over production of APX increased the peroxidase activity that enhanced active oxygen scavenging systems, resulting in oxidative stress tolerance and oomycete pathogen resistance (Sarowar et al., 2005). The activity of APX was higher in *B. cinerea* infected leaves of tomato. Few contradictory results on APX are also reported in barley-powdery mildew pathosystem with regard to APX activity. The study showed a substantial APX activity increase in susceptible barley cultivars and a less pronounced increase in the resistant cultivar after powdery mildew infection. Whereas Venacker et al. (1998) using same pathosystem showed that the foliar APX activity decreased in the resistant isoline, while it remained unchanged in the susceptible one.

2.4.8. Glutathione reductase (GR)

Glutathione reductase is a flavonoid protein oxidoreductase, which participates in the H_2O_2 scavenging pathway (Smith et al., 1989). GR catalyses the conversion of oxidized glutathione (GSSG) to its reduced form (GSH), and plays a crucial role in AOS scavenging (Meister and Anderson, 1983). The three enzymes, glutathione reductase, dehydroascorbate reductase (glutathione dehydrogenase) and ascorbate peroxidase catalyase reactions that maintain large pools of GSH and ascorbate in the chloroplast and channelize reducing equivalents from NADPH to H_2O_2 (Smith et al., 1989). Scavenging of oxidized glutathione by chloroplastic GR is essential in maitaining oxidized glutathione balance by chroloplastic (Foyer et al., 1995). The H_2O_2 scavenging in particular is carried out by catalase, various peroxidases and the ascorbate-glutathione pathway (Halliwell-Asada pathway). Overexpression of GR gene in powdery mildew susceptible wheat enhanced host resistance to *Blumeria graminis*. Waller et al. (2005), showed that *P. indica* infested barley is highly resistant to abiotic and biotic stresses by reprogrammed metabolic state, which includes an enhanced antioxidant capacity and an activation of glutathione-ascorbate cycle.

2.5. Proteomic approaches to study the host responses against the powdery mildew infection

It has been shown that resistance determinants were associated with both pathogen entry into plant host cells and prevention of the spread of the mycelium in the plant (Hardoim et al., 2015). In plants, resistance is achieved by efficient cell wall architecture and related chemical barriers, and an inducible defense mechanism that is excited upon pathogen attack (Durrant and Dong, 2004; Ferrari et al., 2007; Truman et al., 2013). In order to understand

and decipher the plant protection against the pathogen, it is necessary to study the factors contributing to the resistance, following the post infection events.

In the past few years, plant proteome analysis has significantly exploded the knowledge in several physiological and stress related mechanisms including plant-pathogen interactions (Nat et al., 2007; Quirino et al., 2010; Delaunois et al., 2014; Gupta et al., 2015). Proteomics, a comprehensive and quantitative analysis of proteins that are expressed in a given organism under a given situation provides an understanding of the biological process that cannot be obtained by genomics or transcriptomic approaches (Wolters et al., 2001; Gerster et al, 2010). Advances in mass spectrometry and progress in bioinformatics have accelerated the proteomic approaches to study plant-pathogen interactions (Quirino et al., 2010).

Quantitative proteomics methods are broadly classified into two major categories i.e. labeling and label free quantification. The major labeling methods include stable isotope labeling by amino acid in cell culture (SILAC) (Ong et al., 2002), tandem mass tags (TMT) (Thompson et al., 2003), Isotope-coded affinity tags (ICAT) (Gygi et al., 1999) and isobaric tags for relative and absolute quantification (iTRAQ) (Ross et al., 2004). The complexity in sample preparation, the requirement for high concentration of the sample and incomplete labeling are the major drawbacks of labeling methods (Patel et al., 2009). Label free quantification methods have become important techniques in quantitative proteomics approach because of the reproducibility (Wang et al., 2008). Label free quantification is based on the principle of comparison of the peptide peak areas between LC-MS runs without any isotopic labeling (Bondarenko et al., 2002; Griffin et al., 2010). Intensities of peptide peaks are shown to linearly correlate with the quantities of the respective proteins in the sample (Wang et al., 2008). In sunflower, label free quantification was successfully employed in heterosis profiling of sunflower leaves (Mohayeji et al., 2014) and cold acclimation (Balbuena et al., 2011). The technique was also used efficiently in understanding the Piper-*phytophthara* interaction (Mahadevan et al., 2016) and *Arabdopsis*-virus infection (Niehl et al., 2013).

In sunflower, proteomes were analyzed for abiotic stresses like heavy metal stress (Walliwalagedara et al., 2010; Lopes et al., 2015), drought acclimation (Castillejo et al., 2008; Fulda et al., 2011), cold acclimation (Balbuena et al., 2011), heterosis (Mohayeji et al., 2014; Alireza, 2014) and seed quality traits (Baudet and Mosse, 1997; Hajduch et al., 2007; Furse et

al., 2013) (Table 2.4). One study reported the proteomic analysis of sunflower in response to *Sclerotinia sclerotiorum* fungus infection (Akram et al., 2015). Different donors conferring resistance to powdery mildew infection showed different levels of resistance. Proteomic analysis would provide candidate proteins and protein pathways involved in the resistance mechanism. Studies with barley (Godfrey et al., 2009; Noir et al., 2009; Bindschedler et al., 2009; 2011), wheat (Li et al., 2011; Oberhaensil et al., 2011; Wang et al., 2012; Liu et al., 2013), pea (Curto et al., 2006) and grapevine (Marsh et al., 2010) have contributed significantly in defining the specific function of proteins involved in the powdery mildew interaction in these crops.

Table 2.4 List of traits which were analyzed for their proteomes in sunflower

Trait	Method	Important findings	Reference
Seed Oil	2D-PAGE and MALDI-TOF mass spectrometry	Suggested seed oil content is tightly linked to carbohydrate metabolism and protein synthesis in a complex manner.	Hajduch et al., 2007
Oil bodies	Gel-electrophoresis-chromatography-tandem mass spectrometry	The proteome is rich in oleosins (78 %) with the remainder being made up of caleosins and steroleosins	Furse et al., 2013
Oil body membrane proteins	2D-PAGE	Increased expression of cruciferina-like protein and dehydrin at 30 DAA stage marks the onset of desiccation. The data has been analyzed and discussed to highlight desiccation stage-associated metabolic events during oilseed development	Anita and Satish, 2015
Drought Stress	2D-PAGE and MALDI-TOF mass spectrometry	In response to drought conditions a general decrease in protein spots corresponding to enzymes of the photosynthesis and carbohydrate metabolism was observed in the more susceptible genotype, suggesting inhibition of the energetic metabolism	Castillejo et al., 2008

Drought stress	2D-PAGE and MALDI-TOF mass spectrometry	These proteins represent general stress proteins induced under drought conditions or proteins contributing to basic carbon metabolism.	Fulda et al., 2011
Drought stress during the flowering stage	Nanoscale liquid chromatography coupled to tandem mass spectrometry for identification of affected proteins.	Differential and line-specific proteomic changes were attributed as the source for contrasting response to drought stress. According to the results, scavenging of reactive oxygen species, conservation of energy and water, and cell-structure integrity constituted the major aspects of drought tolerance in sunflower.	Ghaffari et al., 2017
Metal-ion stress	2D-PAGE and MALDI-TOF mass spectrometry	When Zn or mixed ions solution was added to the substrate, nine proteins showed different expressions. Another twenty-three protein spots also showed considerable variation when both treatments (Zn or mixed ions) were applied. Twelve of these proteins were successfully characterized, six of them being reported for the first time in Helianthus annuus L.	Garcia et al., 2008
Poly metallic constrains	2D DIGE	The study highlighted the sensitivity of the sunflower to a polymeatallic pollution and indicates that its use as remediative tool of trace element polluted soil is limited	Printz et al., 2013
Cytoplasmic male sterility and fertility restorer	2D-PAGE and MALDI-TOF	These results indicated that hybridization between two parental lines can cause expression differences between sunflower hybrid and its parents not only at mRNA levels but also at protein abundances	Shabani, 2017
Salt stress	SDS-PAGE	The effect of salt were positive with appearance of two bands (in 250 mM before and after 18.5 kDa for Abendsoone	Messaitfa et al., 2014

		variety) not observed in control	
Heterosis	label free LC-MS/MS	The numbers of 38 proteins significantly showed heterosis pattern. The comparison between hybrid and parental inbred lines showed that hybrid vigor is actually linked by emphasize in assimilation rate and low energy consuming.	Mohayeji et al., 2014
Seed protein	2D-PAGE	A quantative study indicated that albumin, globulin, protein and glutelin fractions amounted 38.22, 39.04, 5.53 and 17.09 % respectively of the extracted proteins	Shabani, 2014
Pollen allergies	Mass spectrometry	In this study, the allergenicity of sunflower (*Helianthus annuus*) pollen grains were primarily investigated by clinical studies followed by detailed immunobiochemical and immunoproteomic analyses.	Ghosh et al., 2016
Resistance to parasitic weed Orobanche cumana	iTRAQ	Investigated the differential proteins and pathways between resistant and susceptible cultivars to reveal the mechanisms underlying their different reactions against *O. Cumana*	Yang and Chong, 2017

2.6. Genetic approach to study the inheritance and molecular mapping of powdery mildew resistance gene(s)

After identifying the most promising sources of resistance to powdery mildew, the next important step is to study the inheritance and mapping of gene(s) responsible for powdery mildew resistance in sunflower. Though much work was not done on the inheritance of powdery mildew resistance in sunflower, a few reports are available (Jan and Chandler, 1988; Rojas-Barros et al., 2006; Christov, 2008; Nagayya, 2013). A germplasm (PM1) was reported as resistant to powdery mildew and BC_2F_1 population study showed that powdery mildew resistance was governed by a partially dominant gene (Jan and Chandler, 1988). Rojas-Barros et al. (2006) suggested two gene control of inheritance of powdery mildew resistance in two crosses of *H. argophyllus* X HA 89 and *H. debilis* X HA 89 and use of a high number of the

population might be required for further study of the polygenic inheritance of powdery mildew resistance. Christov (2008) proposed two types of inheritance of powdery mildew resistance in sunflower. In one type, it was controlled by a dominant gene (species like *H. decapetalus* exhibit this type of inheritance), whereas in another type, it was polygenic (species like *H. glaucophyllus, H. ciliaris, H. laevigatus, H. debilis, H. tuberosus* and *H. resinosus*). Nagayya (2013) studied the inheritance of powdery mildew resistance by using five crosses and showed that resistance in few crosses was controlled by single dominant or recessive genes. This study reported that the irregular frequency distribution curves of powdery mildew disease incidence might be contributed by more than one gene.

To the best of the knowledge available, there are limited reports on mapping the powdery mildew resistance in sunflower. Inheritance and mapping studies were reported in other economically important crops (Table 2.4). A few QTLs were mapped for powdery mildew resistance on different crops based on the phenotypic observation of resistance on the segregation population (Heun et al., 1990; Masterbroek, 1991; Czembor and Czember, 2000; Foulongne et al., 2003; Rashid, 2005; Shtaya et al., 2006; Calgene and Durel, 2006; Reddy 2009; Ning et al., 2014) but it was complicated and environment dependent. Different DNA markers were employed in mapping the powdery mildew resistance in other crops (Table 2.4). The utilization of molecular markers for gene mapping have accelerated the crop breeding, particularly microsatellite markers were widely used in mapping studies due to their codominance and abundance across the genome.

In sunflower, several reports exist on mapping important diseases like downy mildew, rust and sclerotium (Table 2.5). An efficient marker assisted selection involves the study of inheritance of resistance and identification of the location of resistance genes on the chromosome (gene mapping). Among the markers utilized for mapping different biotic stress in sunflower, the most widely used markers are the simple sequence repeats (SSRs) (Table 2.5).

Table 2.4 Powdery mildew resistance genes / QTLs mapped in other crops

Crops	Pathogen	Population	Inheritance	Markers used	Reference
Apple	*Podosphaera leucotricha*	F_1	One of the QTL	SSRs	Stankiewicz-Kosyl et al., 2005
Apple	*Podosphaera leucotricha*	F_1	Seven additive and/dominant QTLs	Field assessments	Calenge and Durel, (2006)
Barley	*Erysiphe graminis* f. sp. *Horde*	Genotype	One dominant gene	Phenotype	Mastebroek, (1991)
Barley	*Erysiphe graminis*	DH	One major QTL	RFLP	Backes et al., 1996
Barley	*Erysiphe gra! Minis DC* f. sp. *hordei Em Marchal*	Land races	Four different resistance alleles	Phenotype	Czembor, (2000)
Barley	*Blumeria graminis* f. sp. *Hordei*	RILs	Four QTLs	Phenotype	Shtaya et al., 2006
Barley	*Blumeria graminis* f. Sp. *Hordei L*	Introgression lines	One QTL	SSR	Schmalenbach et al., 2008
Cucumber	*Podosphaera xanthii*	RILs	One QTL	STS	Sakata et al., 2006
Cucumber	*Podosphaera xanthii and Golovinomyces cichoracearum*	F_6	Four QTLs	SRAPS, SSR, SCARs, STS	Liu et al., 2008
Cucumber	*Podosphaera Fusca*	$F_{2:3}$	Four QTLs	SSR	He et al., 2013
Cucumber	*Podosphaera fusca (synonym Podosphaera xanthii)*	F_2	Major QTL	SSR	Xu et al., 2016
European Peach orchards	*Sphaerotheca pannosa var. Persicae*	F_1	Five QTLs	Phenotype	Foulongne et al., 2003
Flax	*Oidium lini*	F_2	Three QTL	Phenotype	Asgarinia et al., 2013
Grapevine	*Erysiphe necator*	Five populations	One QTL	SSR	Riaz et al., 2011
Grapevine	*Erysiphe necator*	F_1	Major QTL	SSR	Van Heerden et al., 2014
Melon	*Erysiphe cichoraceum*	F_3	Dominant monogenic resistances	Phenotype	Anagnostou et al., 2000
Melon	*P. xanthii*	F_2, BC_1 and	Two	Phenotype	Yuste-lisbona

		BC_2	independent genes		et al., 2010
Melon	*Podosphaera Xanthii*	RILs	Two QTLs	SSRs	Fukino et al., 2008
Melon	*Podosphaera xanthii*	F_2 Population	Major QTL	AFLP, RAPD, SSR, SCAR/CAPS /DCAPS, and Two phenotypic traits	Yuste-lisbona et al., 2011
Melon	*Podosphaera xanthii*	BC_1	QTLs	SSR	Ning et al., 2014
Mungbean	*Erysiphe Polygoni DC*	F_1, F_2 and F_3 population	Two dominant genes	Phenotype	Reddy et al., 1994
Mungbean	*Erysiphe polygoni*	RILs	Single QTL	RFLP	Humphry et al., 2003
Mungbean	*Erysiphe polygoni* DC	F_2 and F_3	Single dominant gene	Phenotype	Reddy, 2009
Oat	*Blumeria graminis* D.C. *(speer)* f. sp. *avenae Em.*	F_3	Dominant gene	SSR, AFLP-derived PCR based markers, STS	Yu and Herrmann, 2006
Pea	*Erysiphe pisi*	Inbred lines	Single recessive gene	Phenotype	Tiwari et al., 1997
Pepper	*Leveillula taurica*	Doubled-haploid	Seven QTLs	1 phenotype, 32 RFLP, 27 RAPD and 74 AFLP markers	Lefebvre et al., 2003
Roses	*Podosphaera pannosa*	Selective genotyping	28 QTLs	AFLP, RGA	Linde et al., 2006
Roses	*Podosphaera pannosa*	Diploid Rose population	Nine QTL loci	AFLP, SSR	Hosseini Moghaddam et al., 2012
Ryegrass	*Blumeria graminis*	F_2	Two QTL	RGA	Schejbel et al 2008
Tomato	*Oidium lycopersici*	F_2	Three QTLs	AFLP	Bai et al., 2003
Tomato	*Oidium neolycopersici*	Advanced backcross populations	Major QTLs	Fine mapping	Faino et al, 2012
Watermelon	*Podosphaera xanthii*	F_2, $F_{2:3}$, and reciprocal backcross	A single incomplete dominant	RAPD, CAPS-G/C, SNP	Kim et al., 2013

			gene		
Wheat	*Erysiphe graminis* f. sp. *tritici*	F_2/F_3	Additive effects, QTL	Phenotype	Hautea et al., 1987
Wheat	*Frisiphe graminis* i. sp. *tritici*	F_2	One dominant resistant	Phenotype	Heun et al, 1990
Wheat	*Erysiphe graminis*	F_2	Monogenically recessive inheritance	Phenotype	Schneider et al., 1991
Wheat	*Blumeria graminis* f. sp. *tritici*	DH	Five QTLs	RFLP	Chantret et al., 2000
Wheat	*Blumeria graminis* f. sp. *tritici*	DH	Two QTLs	RFLP	Mingeot et al., 2002
Wheat	*Blumeria graminis* f. sp. *tritici*	F_1, F_2, F_3 and BC_1	A single dominant gene	SSR	He et al., 2009
Wheat	*Blumeria graminis* f. sp. *tritici*	RILs	Three major QTLs	SSR, AFLP	Muranty et al., 2009
Wheat	*Blumeria graminis* f. sp. *Tritici*	Double haploid	Three QTLs	SSR	Asad et al., 2014
Wheat	*Blumeria graminis* f. sp. *tritici*	$F_{2:3}$	A single dominant gene	SCAR, SSR	Ma et al., 2015

Table 2.5 Resistance genes / QTLs mapped in sunflower for important diseases

Pathogen	Population	Inheritance	Markers used	Reference
Plasmopara halstedii	F_3	*PI_1* and *PI_6*	RFLP	Vear et al., 1997
Plasmopara halstedii	F_3 and F_4	*PI_5* locus	RFLP AFLP	Bert et al., 2001
Plasmopara halstedii	RILs	QTL	AFLP	Al-Chaarani et al., 2002
Plasmopara halstedii	Inbred lines	Single	Phenotype	Rahim et al., 2002
Plasmopara halstedii	Inbred lines	BC_1F_1	Phenotype	Molinero-Ruiz et al., 2002
Plasmopara halstedii	Isogenic inbred lines, open pollinated populations, land races, nine haplotypes	Gene candidates	IFLP	Slabaugh et al., 2003
Plasmopara halstedii	F_3	Single gene	SSR, TIR-NBS-LRR, CAPS	Pankovic et al., 2007
Plasmopara halstedii	Inbred lines	QTL	Phenotype	Tourvieille et al., 2008
Plasmopara halstedii	F_3	*PI_{13}*	SSR	Mulpuri et al., 2009
Plasmopara halstedii	RILs	QTL	SSR–SNP	Vincourt et al., 2012
Plasmopara halstedii	F_2	*PI_{16}*	SSR	Liu et al., 2012
Plasmopara halstedii	$BC_1F_{2:3}$	Dominant, single gene	SSR	Qi et al., 2015 a
Plasmopara halstedii	$F_{2:3}$	*PI_{17}*	SSR	Qi et al., 2015 b
Puccinia helianthi	F_2	*R_2*	SSR	Lawson et al., 2011
Puccinia helianthi	$F_{2:3}$	*R_4*	SCAR	Qi et al., 2011
Puccinia helianthi	F_2	*R_5*	SSR	Qi et al., 2012a
Puccinia helianthi	F_2	*R_{11}*	SSR	Qi et al., 2012b
Puccinia helianthi	$F_{2:3}$	*R_{13a}* and *R_{13b}*	SSR and Indel	Gong et al., 2013
Puccinia helianthi	$F_{2:3}$	*$RHAR_6$*	SSR	Bulos et al., 2013
Puccinia helianthi	F_2	R_4, R_5, R_{13a}, and R_{13b}	SSR and SNP	Qi et al., 2015
Puccinia helianthi	F_2	R_{14}	SSR/indel and SNP	Zhang et al., 2016
Sclerotinia sclerotiorum	F_3	QTL	SSR	Micic et al.,

	families			2005a
Sclerotinia sclerotiorum	RILs	QTL	SSR	Micic et al., 2005b
Sclerotinia sclerotiorum	Inbred lines	QTL	Haplotype polymorphisms in 16 candidate genes	Fusari et al., 2012
Sclerotinia sclerotiorum	Association mapping population	Candidate gene association	Haplotype polymorphisms in 16 candidate genes (SNPs)	Talukder et al., 2016

Taking a cue from all the published reports, the present study was undertaken with objectives to develop a new scoring scale for screening sunflower germplasm against powdery mildew, identify the genus causing powdery mildew in sunflower (India), identification of reliable sources resistance against powdery mildew, study the infection process of powdery mildew in sunflower, unravel the histochemical, biochemical and proteomic changes that occur during *G. orontii* infection process in in both susceptible and resistant sunflower genotypes against *G. orontii* for determining the possibility of their role in conferring host defense in response to powdery mildew infection. The study also aims at investigating the inheritance and mapping the powdery mildew resistance gene(s)

Chapter 3

Materials and methods

3.1. Development of a scoring scale for powdery mildew (*Golovinomyces orontii*) disease and identification of resistant sources in cultivated and wild sunflowers

3.1.1. Plant material

The plant material used in the study included 59 accessions of wild *Helianthus* species being maintained at the ICAR- Indian Institute of Oilseeds Research, Hyderabad (IIOR), India (Table 3.1), cultivar germplasm lines that included prebred lines, a core subset, exotic lines and interspecific derivatives. Prebred lines consisted of 195 lines developed from annual species at the IIOR (Table 3.2). The core subset of cultivated sunflower was obtained from USDA-ARS, Ames, USA and consists of 112 accessions representing 38 countries of origin (Table 3.3). The core subset included two ornamental accessions, seven breeding lines, 12 land races and 91 cultivars (Seiler and Jan, 2010). This subset represents wide variability for several qualitative and quantitative traits. Nineteen exotic lines with differential reaction for biotic stresses like rust and downy mildew were also included. Additionally 35 interspecific derivatives developed at USDA-ARS, Fargo, USA and derived from 12 different wild species, including perennial species and different ploidy groups were screened (Table 3.4).

Table 3.1 List of *Helianthus* species screened against powdery mildew under natural and artificial infection conditions

Species	Accession no	PI number	Plant habit	Ploidy
H. agrestis Pollard	AGR-1567	PI 468414	A	D
H. argophyllus	ARG-19	-	A	D
H. bolanderi	BOL-1023	PI 649867	A	D
H. debilis ssp. *debilis* Nutt.	DEB-689	PI 468689	A	D
	DEB-692	PI 468692	-	-
H. petiolaris ssp. *petiolaris* Nutt.	PET-550	PI 435825	A	D
H. praecox ssp. *praecox* Engelm. & A. Gray		PI 494600	A	D
	PRA-1823			
	PRA-1824	PI 494601	-	-
H. angustifolius	ANG-1241	PI 468419	P	D
H. atrorubens	ATR-1902	PI 503206	P	D
	ATR-1581	-		
H. decapetalus L.	DEC-01	-	P	D, T
	DEC-1887	-	-	-
	DEC-1922	-	-	-
	DEC-2054	-	-	-
	DEC-970	PI 649970	-	-
H. divaricatus L.	DIV-1881	-	P	D

	DIV-1885	-	-	-
H. eggertii Small.	EGG-1629	-	P	H
H. giganteus L.	GIG-184	PI 547184	P	D
	GIG-2014	-	-	-
	GIG-2015	-	-	-
	GIG-2017	-	-	-
	GIG-984	PI 649984	-	-
H. grosseserratus Mar.	GRA-10	-	P	D
	GRA-2043	-	-	-
	GRA-2212	-	-	-
	GRA-977	-	-	-
H. hirsutus Raf.	HIR-03	-	P	T
	HIR-1536	-	-	-
H. laevigatus T. & G.	LAE-5709	PI 435709	P	T
H. maximiliani Schr.	MAX-007	PI 650007	P	D
	MAX-010	PI 650010	-	-
	MAX-11	PI 650011	-	-
	MAX-1631	-	-	-
	MAX-2010	-	-	-
	MAX-30	-	-	-
	MAX-33001	-	-	-
H. microcephalus T.&G.	MIC-1872	PI 503231	P	D
H. mollis Lam.	MOL-1530	-	P	D
	MOL-1629	-	-	-
H. nuttallii ssp. *nuttallii* T. & G.	NUT-023	PI 650023	P	D
	NUT-05	-	-	-
	NUT-1517	-	-	-
H. occidentalis ssp. *occidentalis* Riddell.	OCC-52	-	P	D
H. pauciflorus	PAU-S-869	PI 435869	P	H
H. resinosus Small.	RES-09	-	P	H
H. rigidus	RIG-1696	-	P	H
H. salicifolius Dietr.	SAL-241	PI 435872	P	D
H. strumosus L.	STR-15	-	P	D, H
	STR-1934	-	-	-
	STR-216	PI 547216	-	-
	STR-251	PI 503251	-	-
H. tuberosus L.	TUB-05	-	P	H
	TUB-07	-	-	-
	TUB-15	-	-	-
	TUB-1705	-	-	-
	TUB-2069	PI 547243	-	-
	TUB-2729	AMES2729	-	-

Ploidy: D = diploid (n = 17), T = tetraploid (n = 34), H = hexaploid (n = 51). Growth habit: A= annual, P = perennial.

Table 3.2 List of prebred lines screened against powdery mildew under natural and artificial infection conditions

PS 1001-2000 Series			PS 2001-3000 Series		PS 3001-4000 Series	PS 4001-5000 Series			PS 5001-6000 Series	PS 6001-7000 Series
PS-1028	PS-1003	PS-1022	PS-2029	PS-2041	PS-3005	PS-4091	PS-4024	PS-4091-2	PS-5011-B	PS-6002
PS-1070	PS-1005	PS-1027	PS-2037	PS-2042	PS-3010	PS-4037	PS-4026	PS-4112	PS-5032	PS-6009
PS-1002	PS-1006	PS-1029	PS-2064	PS-2046	PS-3028	PS-4015	PS-4027	PS-4003	PS-5003	PS-6004
PS-1026	PS-1015	PS-1032	PS-2065	PS-2051	PS-3005	PS-4050	PS-4066	PS-4006	PS-5011-1	PS-6011
PS-1027	PS-1020	PS-1034	PS-2001	PS-2056	PS-3008	PS-4052	PS-4114	PS-4007	PS-5014	PS-6008
PS-1053	PS-1023	PS-1036	PS-2003	PS-2057	PS-3009	PS-4008	PS-4004	PS-4039	PS-5015	PS-6015
PS-1059	PS-1078	PS-1037	PS-2005	PS-2025	PS-3011	PS-4017	PS-4015-1	PS-4050	PS-5017	
PS-1063	PS-1033	PS-1038	PS-2006	PS-2027	PS-3002	PS-4019	PS-4018	PS4051	PS-5018	
PS-1064	PS-1049	PS-1039	PS-2009	PS-2028	PS-3030	PS-4023	PS-4001	PS-4055	PS-5006	
PS-1067	PS-1051	PS-1040	PS-2013	PS-2043	PS-3033	PS-4028	PS-4005	PS-4057	PS-5010	
PS-1068	PS-1081	PS-1041	PS-2016	PS-2062	PS-3034	PS-4034	PS-4033	PS-4059	PS-5019	
PS-1071	PS-1086	PS-1043	PS-2020	PS-2059	PS-3038	PS-4036	PS-4034	PS-4060	PS-5007	
PS-1075	PS-1014	PS-1047	PS-2021	PS-2026	PS-3035	PS-4043	PS-4038	PS-4068	PS-5020	
PS-1080	PS-1015	PS-1050	PS-2023	PS-2058	PS-3004	PS-4045	PS-4047	PS-4070	PS-5022	
PS-1084	PS-1017	PS-1062	PS-2024			PS-4051	PS-4003	PS-4071	PS-5023	
PS-1087	PS-1018	PS-1082	PS-2030			PS-4002	PS-4061	PS-4074	PS-5024	
PS-1091	PS-1020		PS-2033			PS-4010	PS-4069	PS-4079	PS-5026	
PS-1095	PS-1021		PS-2040			PS-4020	PS-4088-1	PS-4091-2		
								PS-4091-2		
								PS-4091-2		

*Prebred lines in 1000 series are derived from *H. argophyllus* x cult. sunflower; 2000 series from *H. petiolaris* x cult. sunflower ; 3000 series from Wild *H. annuus* x cult. sunflower ; 4000 series from *H. argophyllus* x Wild *H. annuus* x cult. sunflower – trispecific; 5000 series from cult. sunflower x *H. debilis;* 6000 series from cult. sunflower x *H. maximiliani*.

Table 3.3 List of core germplasm lines screened against powdery mildew under natural and artificial infection conditions

IIOR Identity	EC No	Plant name	IIOR Identity	EC No	Plant name
SCG-1	EC625687	Sundak	SCG-35	EC625721	SENECA
SCG-2	EC625688	SUNRISE	SCG-36	EC625722	VISHOD
SCG-3	EC625689	No. 2770	SCG-37	EC625723	PEREDOVIK304 USSR 60% OIL
SCG-4	EC625690	No.3332	SCG-38	EC625724	SMENA
SCG-5	EC625691	AYCICEGI	SCG-39	EC625725	VNIIMK 6540
SCG-6	EC625692	GONONDU	SCG-40	EC625726	DARK STRIPE
SCG-7	EC625693	NOVOSAKSKI BR.4	SCG-41	EC625727	KENYA WHITE
SCG-8	EC625694	No. 9588	SCG-42	EC625728	GUAYACANINTA
SCG-9	EC625695		SCG-43	EC625729	IMPIRA INTA
SCG-10	EC625696	AFTAB-PARAST	SCG-44	EC625730	ARGE PEHUGEN
SCG-11	EC625697	No. 2	SCG-45	EC625731	HATZOR AYALA
SCG-12	EC625698	LOVASZPATONAI	SCG-46	EC625732	VIR 847
SCG-13	EC625699	W.Y.1/7	SCG-47	EC625733	RELAX HYBRID GERMPLASM
SCG-14	EC625700	JDANOVSKY6432 ND 3156OIL	SCG-48	EC625734	KORTUS
SCG-15	EC625701	No.k1879	SCG-49	EC625735	CAKINSKIJ 321
SCG-16	EC625702		SCG-50	EC625736	PROGRESS
SCG-17	EC625703		SCG-51	EC625737	Romsun N-2-2004
SCG-18	EC625704	VNIIMK 16464 MOTHRES	SCG-52	EC625738	43-48-Vk-32
SCG-19	EC625705	CINZA 42	SCG-53	EC625739	Dd-75-4
SCG-20	EC625706	ENISEJ	SCG-54	EC625740	d-75-10
SCG-21	EC625707	No.5	SCG-55	EC625741	NS-B-16-62/1
SCG-22	EC625708	SZARATOVSZKU RANNI	SCG-56	EC625742	Hopi dye
SCG-23	EC625709	JUPITER	SCG-57	EC625743	
SCG-24	EC625710	AGUAPEI	SCG-58	EC625744	
SCG-25	EC625711	ARMAVIRSKY	SCG-59	EC625745	Giza
SCG-26	EC625712	VNIIMK 8883	SCG-60	EC625746	CCA 82-2
SCG-27	EC625713	BLACK SAYAR	SCG-61	EC625747	Pervenets
SCG-28	EC625714	MANFREDI INTA (3-WAY X)I	SCG-62	EC625748	Egnazia
SCG-29	EC625715	USSR MAYAK'66	SCG-63	EC625749	Ames 101
SCG-30	EC625716	USSR VNIIMK 8931'66	SCG-64	EC625750	ames 2350
SCG-31	EC625717	TCHERNIANKA SELECT W-17	SCG-65	EC625751	Damaya

SCG-32	EC625718	W-32	SCG-66	EC625752	Liao 2
SCG-33	EC625719	HAVASUPAI	SCG-67	EC625753	VIR 019
SCG-34	EC625720	HOPI	SCG-68	EC625754	VIR 160

IIOR Identity	EC No	Plant name	IIOR Identity	EC No	Plant name
SCG-69	EC625755	Start	SCG-97	EC625783	VK-47
SCG-70	EC625756	VK-12	SCG-98	EC625784	HS 61
SCG-71	EC625757		SCG-99	EC625785	ROMSUN V3355
SCG-72	EC625758	Odesskij 113	SCG-100	EC625786	371-3 S
SCG-73	EC625759		SCG-101	EC620182	RHA 426
SCG-74	EC625760		SCG-102	EC620183	VIR 107
SCG-75	EC625761	BEKECSI "B"	SCG-103	EC620184	Karlik
SCG-76	EC625762	CSEHSZLOVAKIAI "B"	SCG-104	EC620185	arrowhead
SCG-77	EC625763	FRANCIA "E"	SCG-105	EC620186	Mennonie
SCG-78	EC625764	GEORGIA	SCG-106	EC620187	chang ling
SCG-79	EC625765	KRZYNOWLOSKI MIEJSCOWY	SCG-107	EC620188	ames 10101
SCG-80	EC625766	WIELKOPOLS Ki	SCG-108	EC620189	Sannace
SCG-81	EC625767	CO-PB 68	SCG-109	EC620190	Stepnyak
SCG-82	EC625768	Mandan#1	SCG-110	EC620191	Zaria
SCG-83	EC625769	Hidatsa#2	SCG-111	EC620192	skorospelyi
SCG-84	EC625770	Hemas	SCG-112	EC620193	Kosim
SCG-85	EC625771	Record	SCG-113	EC620194	Ames 21671
SCG-86	EC625772	Sepasol			
SCG-87	EC625773	L-2625-1 (Ukraine)-1			
SCG-88	EC625774	6 sc ug 16			
SCG-89	EC625775	Oleisty Borovskij\			
SCG-90	EC625776	B 4268			
SCG-91	EC625777	Fuksinka 10			
SCG-92	EC625778	Gigant 549			
SCG-93	EC625779	Kvuglik A-41			
SCG-94	EC625780	B-7422			
SCG-95	EC625781	Yawne			
SCG-96	EC625782	Advance			

Table 3.4 List of interspecific derivatives screened agianst *G. orontii* under field (net contained) conditions

IIOR Identity	Germplasm	Reg. no.	PI Number	Pedigree
ID-1	ANO-1509-1	GP-135	PI 539894	cmsHA 89*2/*H. anomalus* (Acc. 1509)//RHA 265 F_4
ID-2	ANO-1509-2	GP-136	PI 539893	cmsHA 89*2/*H. anomalus* (Acc. 1509)//RHA 265 F_4
ID-3	BOL-774	GP-143	PI 539892	cmsHA 89*2/*H. bolanderi* (Acc. 774) F_3
ID-4	DEB-CUC-1810	GP-146	PI 539911	nms P21*3/*H. debilis* ssp. *cucumerifolius* (Acc. 1810) F_3
ID-5	DEB-SIL-367-2	GP-145	PI 539908	cmsHA 89*3/*H. debilis* ssp. *silvestris* (Acc. 367) F_3
ID-6	DES-1474-1	GP-162	PI 539912	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4
ID-7	DES-1474-2	GP-163	PI 539913	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4
ID-8	DES-1474-3	GP-164	PI 539914	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4
ID-9	GIG-1616-1	GP-237	PI 610782	nmsHA P21/*H. giganteus* (Acc. 1616) BC_1F_2
ID-10	GIG-1616-2	GP-238	PI 610783	nmsHA P21/*H. giganteus* (Acc. 1616) BC_1F_2
ID-11	HIR-828-1	GP-239	PI 610784	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2
ID-12	HIR-828-2	GP-240	PI 610785	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2
ID-13	HIR-828-3	GP-241	PI 610786	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2
ID-14	HIR-828-4	GP-242	PI 610787	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2
ID-15	HIR-1734-1	GP-150	PI 539907	cmsHA 89*2/ *H. hirsutus* (Acc. 1734) F_3
ID-16	HIR-1734-2	GP-151	PI 539906	cmsHA 89*2/ *H. hirsutus* (Acc. 1734) F_3
ID-17	HIR-1734-3	GP-152	PI 539905	cmsHA 89*2/ *H. hirsutus* (Acc. 1734) F_3
ID-18	NEG-1255-1	GP-147	PI 539904	nms P21*2/*H. neglectus* (Acc. 1225) F_3
ID-19	PAR-1084-1	GP-138	PI 539901	cmsHA 89*2/*H. paradoxus* (Acc. 1084) F_4
ID-20	PAR-1673-1	GP-160	PI 539899	cmsHA 89*2/*H. paradoxus* (Acc. 1673) F_3
ID-21	PRA-PRA-1142	GP-149	PI 539910	nms P21*2/*H. praecox* ssp.*praecox* (Acc. 1142) F_5
ID-22	PRA-RUN-417-1	GP-156	PI 539886	cms HA 89*/ *H. praecox* subsp. *runyonii* (Acc. 417) F_3
ID-23	RES-834-1	GP-153	PI 539897	cms HA 89*2/*H. resinosus* (Acc. 834) F_5
ID-24	RES-834-2	GP-154	PI 539896	cms HA 89*2/*H. resinosus* (Acc. 834) F_4
ID-25	RES-834-3	GP-155	PI 539895	cms HA 89*2/*H. resinosus* (Acc. 834) F_5
ID-26	STR-1622-1	GP-243	PI 610788	nmsHA P21/*H. strumosus* (Acc. 1622) BC_1F_2
ID-27	STR-1622-2	GP-244	PI 610789	nmsHA P21/*H. strumosus* (Acc. 1622) BC_1F_2
ID-28	TUB-346	GP-181	PI 564549	cmsHA 89*2/*H. tuberosus* (Acc. 346) F_3
ID-29	TUB-365	GP-182	PI 564515	cmsHA 89*2/ *H. tuberosus* (Acc. 346) F_3
ID-30	TUB-825-1	GP-245	PI 610790	nmsHA P21/ *H. tuberosus* (Acc. 825) BC_1F_2
ID-31	TUB-825-2	GP-246	PI 610791	nmsHA P21/*H. tuberosus* (Acc. 825) BC_1F_2
ID-32	TUB-1709-1	GP-183	PI 564517	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_3
ID-33	TUB-1709-2	GP-184	PI 564518	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_4
ID-34	TUB-1709-3	GP-185	PI 564519	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_3
ID-35	TUB-1789	GP-186	PI 564520	cmsHA 89*2/nms P21 VR1*1/*H. tuberosus* (Acc. 1789) F_3

3.1.2. Confirmation of the pathogen

For microscopic observations of the pathogen, infected leaves were scraped gently to dislodge the conidia along with the hyphae. The conidia were stained with lactophenol cotton blue and observed under light microscope (Leitz, Diaplan). The procedures for DNA extraction, primers used and PCR amplification were as described by Chen et al. (2008) with minor modifications. Total genomic DNA was extracted from diseased leaf and mycelia scraped from the infected leaves of the susceptible cultivar, PS 2023 following the standard CTAB method with minor modifications (Doyle and Doyle, 1990). The material was ground in liquid nitrogen, then homogenized in 20 ml of extraction buffer (2% CTAB, 20 mM EDTA, 2% PVP, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0 and 1% β-mercaptoethanol) and incubated at 65 ^{0}C for 1 h. The supernatant was extracted twice with chloroform: isoamylalcohol (24:1 v/v) and treated with RNase A (100 μg/ml), incubated at 37 ^{0}C for 30 min. The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and resuspended in 500 μl of sterile MilliQ water and stored at –20 ^{0}C. The DNA concentration was determined electrophoretically using known amount of λ DNA as standard. The internal transcribed spacer (ITS) of nuclear ribosomal DNA regions was amplified from powdery mildew of sunflower using the powdery-mildew-specific ITS universal primer pair PN 23 (5'-CAC CGC CCG TCG CTA CTA CCG-3')/PN 34 (5'-TTG CCG CTT CAC TCG CCG TT-3') and pair of primers, S1 (5'-GGA TCA TTA CTG AGC GCG AGG CCC CG-3')/S2 (5'-CGC CGC CCT GGC GCG AGA TAC A-3'), G1 (5'-TCC GTA GGT GAA CCT GCG GAA GGA T-3')/G2 (5'-CAA CAC CAA ACC ACA CAC ACG GCG-3'), and L1 (5'-CCC TCC CAC CCG TGT CGA CTC GTC TC-3')/L2 (5'-CTG CGT TTA AGA GCC GCC GCG CCG AA-3'), that were specific to the ITS regions of *P. xanthii*, *G.orontii* (*G. cichoracearum*), and *L. taurica*, respectively. The PCR reaction mixture contained 0.15 mM dNTPs, 0.4 μM primers, 1 U *Taq* DNA polymerase, 1X PCR buffer with 1.5 mM $MgCl_2$ (Bangalore Genei, India), and 10 ng of template DNA and sterile distilled water to a final volume of 25 μl. PCR amplification was carried out in a thermal cycler (Applied Biosystems GeneAmp 9700) using the following amplification conditions: 5 min at 94 ^{0}C for the initial denaturation, followed by 30 cycles consisting of 40 s of denaturation at 94 ^{0}C, 1 min of annealing at 62 ^{0}C, and 1.5 min of extension at 72 ^{0}C with a final extension at 72 ^{0}C for 5 min. The PCR amplified products were resolved by

electrophoresis on 2.0% agarose (Bangalore Genei, India) gel in 1 X Tris-acetate-EDTA buffer by electrophoresis at 100 V for 3 h and visualized with ethidium bromide staining. The gel images were recorded using the Alpha Innotech Fluorchem gel documentation system.

3.1.3. Field evaluation

Screening of *Helianthus* species and cultivar germplasm for reaction to powdery mildew was carried out under natural disease incidence conditions at the research farm of the IIOR, Hyderabad, India during the months of October to March during the years 2010 and 2011 with Morden (early maturing open-pollinated variety), DRSF-108 (population) and the interspecific derivative PS 2023 (derived from *H. petiolaris*) as the susceptible checks. These months had the most favorable conditions for powdery mildew infection with a mean temperature of 24 ^{0}C ranging from 16 ^{0}C to 33 ^{0}C. The relative humidity ranged from 35-74%. The mean sunshine hours were 8, the mean wind speed was 2.9 Km/h with an evaporation rate of 3.2 mm. Each entry was planted in a single row of 5 m length spaced 60 cm apart with a plant-to-plant distance of 20 cm and was replicated twice. At flowering stage when disease symptoms started appearing, the field was enclosed with a white net (1 mm pore size) to enhance the spore load and disease intensity. Disease severity was recorded at weekly intervals from flowering till maturity stage. The leaf area and days to 50% flowering of each line were recorded.

3.1.4. Artificial infection methods to evaluate the best infection procedure

Sources of resistance identified in the cultivated germplasm in the field and all the wild *Helianthus* accessions were screened under controlled conditions, following artificial inoculation. In order to select the most effective and convenient method of infection for artificial screening, seven different methods described by Karuna (2010) were tested on a susceptible variety (Morden) and 2 resistant lines [(RES-843-3 (ID-25) and PI 642072 (TX 16R)]. These methods were

- Dusting method where infected leaves with conidia were dusted on test leaves moistened with water
- Spraying of spore suspension (10^6 conidia/ml) that was prepared by washing infected leaves with sterile water and filtering through double layered cheese cloth

- Cotton swab method in which spore solution (10^6 conidia/ml) was gently applied on healthy test leaves with cotton wool
- Dipping leaves of healthy test plants in spore solution (10^6 conidia/ml) for 5 min;
- Stapling method in which powdery mildew infected leaves were stapled on to healthy leaves and covered with polythene bags overnight;
- Blotter paper method in which blotter paper dipped in the conidial suspension (10^6 conidia/ml) was placed on the test leaves;
- Gentle rubbing of leaves of the healthy plants against infected leaves with conidia.

Care was taken that leaves infected with pathogen were not too old and were not collected under wet weather conditions as it prevents release of conidia from the conidiophores. Among these methods, dusting of spores on test plant leaves proved to be convenient and effective. Hence, the wild species and germplasm lines that showed resistance under field conditions were further evaluated for resistance under artificial conditions by the spore dusting method. About 6-8 weeks after sowing, plants were infested by dusting conidia from heavily infected leaves. Following inoculation, the plants were covered with polythene bags and the relative humidity (RH) of the green house was maintained at 60-70% for 48 h with occasional misting at regular intervals. The polythene bags were removed thereafter and a temperature of 26-30 ºC and RH of 60-70% were maintained throughout the experimental period. Since there were differences in the level of infection in the wild *Helianthus* species, spore counts per unit area were made. For this, three discs of infected leaves were punched with the eppendorf tube (1.5 ml) cap and immersed in 500 µl of sterile water in the same tube. The material was vortexed to dislodge the spores and the supernatant was collected. The process was repeated twice and all the 3 pooled samples were used for spore count. Spore count was determined using a standard haemocytometer and represented as spores/cm^2. The perennial species *H. strumosus* (STR-15) was highly susceptible and harboured the pathogen throughout the year and hence, served as a continuous source of fresh inoculum.

3.1.5. Disease assessment

For scoring the powdery mildew incidence, a disease scoring scale (0-9) was developed (Table. 2.1). With regard to disease infection level, the material was scored as 0= no infection on leaves; low (L) - if leaves had < 10% infection; moderate (M) - if leaves had 11-

30% infection, and high (H) - if leaves had 31-100% infection (Fig. 3.1). Powdery mildew severity was scored at 10-days-interval from 45 days of sowing till plant maturity.

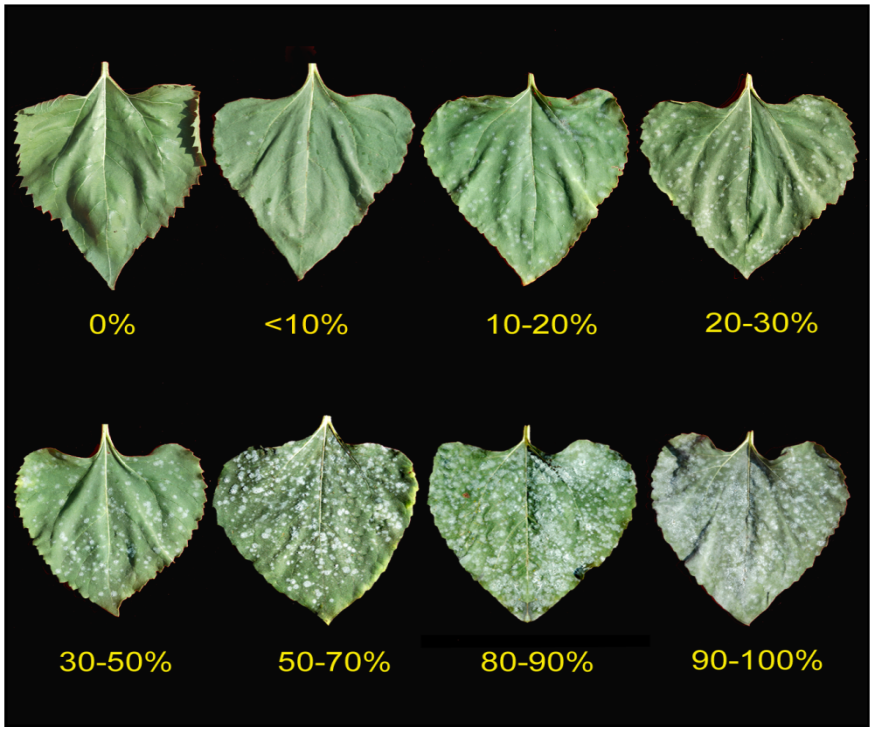

Fig. 3.1. The 0-9 scoring scale for powdery mildew in sunflower is based on the percentage of infection levels on leaves

Table 3.5. A 0-9 scale for scoring powdery mildew incidence in sunflower

Position of leaf	Bottom	Middle	Top	Scale	Category
Severity of powdery mildew	High	High	High	9	Highly susceptible
	High	High	Medium	8	Highly susceptible
	High	High	Low	7	Susceptible
	High	Medium	Medium	6	Susceptible
	High	Medium	Low	5	Susceptible
	High	Low	Low	3	Moderately resistant
	Medium	Medium	Medium	4	Moderately resistant
	Medium	Medium	Low	4	Moderately resistant
	Medium	Low	Low	2	Resistant
	Low	Low	Low	1	Highly resistant
	0	0	0	0	Immune

0 = no infection, Low = < 10% infection, Medium = 11-30% infection, High = 31-100% infection

3.1.6. Statistical analysis

Disease was scored on 15 plants on a 0-9 scale (0 = immune; 1= highly resistant, 2 = resistant; 3-4 = moderately resistant; 5-9 = susceptible/highly susceptible). Disease severity index (DSI) was calculated according to Kim et al. (2000) using the following formula; and the final severity (disease intensity at the final score) is presented.

$$\text{DSI} = \frac{\sum(\text{Ratings for each plant})}{9 \text{ X Number of plants rated}} \text{ X } 100$$

Where Σ is the sum of the disease rating of the plants, 9 is the highest disease rating.

The progression of powdery mildew disease over time was determined as the area under disease progression curve (AUDPC) according to the formula of Campbell and Madden (1990) as follows.

$$\text{AUDPC} = \sum_{i=1}^{n}[(x_i + x_{i-1})/2](t_i - t_{i-1})$$

Where n is the number of evaluation times, x_i is the disease intensity at the evaluation time and (t_i-t_{i-1}) is the duration between each evaluation.

Leaf area (LA) was estimated by the method described by Rouphael et al. (2007).

DSI and AUDPC were calculated using Microsoft Excel software 12.0. Data on spore counts were subjected to analysis of variance (ANOVA) using the SAS 9.3 package (SAS Institute Inc. USA). Each replication had observations from 10 plants and each treatment had three replicates. The treatment means were compared using DMRT at P< 0.001. Correlations between DSI, AUDPC, days to 50% flowering and leaf area were determined by using Pearson correlation coefficient at P < 0.001.

3.2. Histopathological study of the *G. orontii* infection process in sunflower and host defense responses to infection

3.2.1. Plant material and infection process

Based on the differential reaction of the *Helianthus* species and cultivar germplasm to powdery mildew, four genotypes were selected for the study *viz.,* Morden (susceptible), PS 2023 (derived from *H. petiolaris*, highly susceptible), and resistant genotypes, ID-25 (RES-843-3) and TX 16R (PI 642072) (Reddy et al., 2013). All the plant materials were grown in a controlled environment at 24 ^{0}C, 70% relative humidity and a 12 hour light/dark under the greenhouse conditions at the ICAR-Indian Institute of Oilseeds Research, Hyderabad, India. About seven weeks after sowing, plants were infected with *G. orontii* collected by dusting conidia from the highly infected plants of PS 2023 on test leaves moistened with water

according to Reddy et al. (2013). Inoculation densities were kept low (~1000 spores/cm^2) for all the genotypes to discern single colonies on the leaves.

3.2.2. Staining and light microscopy

To determine the epiphytic fungal structures and the stage of fungal development, the leaves of the four genotypes at various time points were stained for microscopic observations. Pathogen-infected leaves were detached at 1 day interval following infection (zero days) up to 10 days and stained with lacto phenol cotton blue followed by decolorizing solution containing ethanol: glacial acetic acid 3:1 (v/v) for $\geq$ 8 hours. Finally, the samples were mounted in 10% glycerol for rehydration as described by Gollner et al. (2008). A light microscope (Eclipse H600L; Nikon, Japan) fitted with a high performance camera (Retiga 2000R), Nikon Digital Sight U3 control unit and image analysis software (NiSElements; Nikon) was used in imaging of samples. The experiment was repeated thrice and 5-10 images were analyzed per replicate, genotype and the time point.

3.2.3. Quantification of *G. orontii* infection by counting of conidiophores

The genotypes, TX 16R and ID-25 were macroscopically resistant against powdery mildew, whereas PS 2023 and Morden were susceptible. To measure relative susceptibility and to determine the level of infection, the number of mature conidiophores per colony was counted after seven days of infection (dpi) through light microscopy. The conidiophores of at least 10 colonies per genotype were counted on leaf samples stained with lacto phenol cotton blue.

3.2.4. Scanning electron microscopy (SEM)

Parts of the infected leaves were collected in vials and fixed with 3% Glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24 hours at 4 ^{0}C and post-fixed with 2% aqueous Osmium tetroxide in the same buffer for 4 hours. Following this, the samples were dehydrated in a series of graded alcohol and dried to critical point drying with Electron Microscopy Science CPD unit. The dried samples were subsequently mounted over the stubs with double sided conductive tape. Finally, a thin layer of platinum metal was applied over the sample using an automated sputter coater (JOEL JFC-1600) for about 3 minutes. The samples were then observed under Scanning Electron Microscope at various magnifications, as per the standard procedures given by John and Russell (1998).

3.2.5. Histochemical localization of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2)

Accumulation of H_2O_2 was detected by 3, 3 diaminobenzidine (DAB) staining. Infected leaves were detached at indicated time points and incubated in 200 µl of DAB (1 mg/ml, pH 3.8) for 8 hours to develop the reddish-brown coloration of the DAB polymer, and bleached in 95% (v/v) boiling ethanol for 10 min as described by Thordal-Christensen et al. (1997). Sub cellular localization of O_2^- in leaves was detected as described by Doke (1983) with minor modifications. The Leaf were vacuum-infiltrated with 0.05 M sodium phosphate buffer (pH 7.5) containing 0.05% nitrobluetetrazolium (NBT) and after 15 min of staining at room temperature under light, the NBT-treated tissues were bleached in 95% (v/v) ethanol to stop reaction and preserve the tissue integrity.

Antioxidant enzyme estimation

3.2.6. Preparation of enzyme extract

All enzyme extractions and centrifugations were carried out at 4 ^{0}C and the extracts were stored at -20 ^{0}C. Control and infected leaves were collected at indicated time points and ground with a pre-chilled sterile mortar and pestle using sand as an abrasive. The homogenate was filtered through a 45 µm nylon mesh and centrifuged at 13,000 g for 20 min at 4 ^{0}C. The supernatant was carefully decanted and used as crude enzyme. All assays were carried out at room temperature and repeated thrice with three biological replicates, per genotype and at each time point.

Superoxide dismutase (SOD; EC 1.15.1.1) and peroxidase (POX; EC 1.11.1.7) were extracted together from a sample of 5 g of control (Un infected) and infected leaves (fresh weight) that was ground in 18 ml of ice cold extraction buffer, which consisted of 0.1 M Tris-HCl buffer (pH 7.5) containing 3% (w/v) polyvinylpyrrolidone, 1 mM EDTA and 1 mM $CaCl_2$. The extraction medium for ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2) and hydrogen peroxide (H_2O_2) consisted of 0.1 M potassium phosphate buffer (pH 7.5) in place of Tris-HCl buffer and the remaining components and procedures were the same.

3.2.7. Hydrogen peroxide (H_2O_2)

The hydrogen peroxide assay solution was prepared with 26 mM of potassium phosphate buffer (pH 7.5) containing 0.2 ml of extract and 2.0 ml of 5% potassium dichromate: acetic acid (1:3 v/v) in 3.0 ml of the final volume, following the

method of Sinha (1972). The contents were mixed well, boiled for 10 min, cooled and absorbance was read at 570 nm against the reagent blank. The quantity of H_2O_2 was determined from the standard curve (20 to 200 µmol) using an equation y=0.0061x (Sinha, 1972). The results were expressed in terms of nmol g^{-1}f.wt.

3.2.8. Superoxide dismutase (SOD; EC 1.15.1.1)

Superoxide dismutase activity was estimated by the inhibition of nitroblueterazolium (NBT) reduction adopting the method of Beauchamp and Fridovich (1971). Each reaction mixture contained 14 mM L-methionine, 60 µM NBT, 3 µM riboflavin, 0.1 mM EDTA, 0.1 ml of enzyme extract and 50 mM Tris-HCl (pH 7.8) to give a final volume of 3 ml. Reaction was initiated by adding riboflavin and continued for 40 min below 30 cm of two 36 W fluorescent lamps (Phillips, India). After the incubation period, tubes were covered with black cloth for protection against light. A non-irradiated reaction mixture that did not develop colour served as the control. The reaction mixture without enzyme extract developed maximum colour and its absorbance decreased with the addition of the enzyme. The absorbance at 560 nm was determined continuously, and the extract volume causing a 50% of photo-reduction inhibition of NBT was taken as one unit of activity. The percent inhibition was calculated according to Asada et al. (1974)

3.2.9. Peroxidase (POX; EC 1.11.1.7)

The reaction of peroxidase was determined by the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm (Rao et al., 1996). The reaction mixture (3.0 ml) contained 2.15 ml of 0.1 M Tris-HCl buffer (pH 7.0), 0.6 ml of 1% guaiacol, 0.1 ml of enzyme extract and 0.15 ml of 100 mM H_2O_2. H_2O_2 was added at the end, mixed by inversion and absorbance was read at 470 nm at 15 sec interval up to 3 min. The linear portion of optical density (OD) change was taken to calculate the enzyme activity using a molar extinction coefficient of 26.6 mM cm^{-1} for guaiacol oxidation. One unit of peroxidase activity was defined as the amount of enzyme required to oxidize one nmol of guaiacol/ min/ ml.

3.2.10. Ascorbate peroxidase (APX; EC 1.11.1.11)

Ascorbate peroxidase was assayed according to the method of Nakano and Asada (1981). The 3.0 ml of reaction mixture contained 95 mM potassium phosphate buffer (pH 7.0), 0.5 mM L-ascorbate and 0.5 mM H_2O_2. The reaction was initiated

by the addition of 50 µl of enzyme extract. The ascorbate peroxidase consumption was monitored by the reduction of absorbance at 290 nm for 2 min against the reagent blank, which corresponded to the oxidation of ascorbic acid. The enzyme activity was calculated using the molar extinction coefficient of 2.8 mM cm^{-1}. One enzyme unit was expressed as the amount of enzyme required to oxidise one nmol of ascorbate/ min.

3.2.11. Glutathione reductase (GR; EC 1.6.4.2)

Glutathione reductase was determined by using the procedure of Halliwell and Foyer (1978). Each reaction mixture contained 816 µM EDTA, 0.2 mM oxidized glutathione, 0.1 mM NADPH, 50 µl of enzyme extract and 82 mM of potassium phosphate buffer (pH 7.5) giving a final volume of 3 ml. The reaction was initiated by adding NADPH and the decrease in absorbance was monitored at 340 nm against 3.0 ml potassium phosphate buffer (0.1 M, pH 7.5) as blank. An extinction coefficient of 6.12 mM cm^{-1} was used to calculate the amount of NADPH oxidized. One enzyme unit was defined as the amount of enzyme required to oxidize one nmol of NADPH/min.

3.3. Differential proteomic analysis following powdery mildew infection in sunflower

3.3.1. Plant material

The plant material used in the study included one susceptible genotype (PS 2023), one resistant/tolerant genotype (TX 16R (PI 642072)) and an immune genotype/species (*H. praecox* ssp. *praecox,* Accn No 1823) (Reddy et al., 2013; Chander Rao et al., 2015).

3.3.2. Method of infection

About 6 to 8 weeks after sowing, plants were infested by dusting conidia of powdery mildew from heavily infected leaves. Following inoculation, the plants were covered with polyethylene bags and the relative humidity (RH) of the greenhouse was maintained at 60 to 70% for 48 h with occasional misting at regular intervals. The polyethylene bags were removed thereafter and a temperature of 26-30 ^{0}C and RH of 60-70% were maintained throughout the experimental period. Three leaves from the three different genotypes were collected 3 days post infection (dpi), 7 dpi, and 10 dpi. Control leaves were treated with

water and maintained under the same conditions as inoculated leaves, and collected at the same time points.

3.3.4. Protein extraction

Three leaves, one each from different genotypes were pooled and the tissue was ground to fine powder with mortar and pestle in liquid nitrogen. One gram of the homogenized powder was resuspended in ice cold TCA-2 mercaptoethanol (2ME)-acetone solution (Mechin et al., 2007), mixed well and stored at -20 ^{0}C for 1 hour. Samples were centrifuged at 10000 g for 10 min at 4 ^{0}C. The supernatant was discarded and rinsing solution containing 0.07% 2ME (v/v) in ice cold acetone was added to the pellet and stored at -20 ^{0}C for 1 hour followed by centrifugation at 10,000 g for 15 min at 4 ^{0}C. The resultant supernatant was discarded and this step was repeated twice and finally the pellets were dried under vacuum for about 1 hour. The pellets were dissolved in solubilization buffer containing 30 mM Tris, 2 M thiourea, 8 M urea, and 4% CHAPS (w/v) at pH 8.8 and vortexed for 1 min followed by centrifugation for 15 min at 10,000 g and the supernatant was collected and it was centrifuged again for 15 min and finally the supernatant containing solubilized proteins was stored at -80 ^{0}C. Protein concentration was estimated by the Bradford method (Bradford, 1976).

3.3.5. In solution digestion and desalting

Total protein of 200 µg was taken and precipitated using methanol-chloroform (v/v) and resuspended in a buffer containing 8 M urea in 100 mM ammonium bicarbonate (pH 8.0). The proteins were reduced with 50 mM dithiothreitol (DTT) for 30 min at room temperature and then alkylated with 50 mM iodoacetamide for 45 min in dark. The proteins were then digested with trypsin at 37 ^{0}C for 12 h at a concentration of 25:1 (w/w) (Promega Trypsin gold). After digestion, the solution was acidified with trifluoroacetic acid (TFA) to quench the action of trypsin and digested peptides were desalted by loading on to the C_{18} STAGE tips (Thakur et al., 2011). Following this, the peptides were eluted using buffer A* (2% acetonitrile, 0.5% formic acid) and proceeded for LC-MS/MS.

3.3.6. LC-MS/MS analysis

Trypsin-digested peptides were loaded on a 15 cm long Thermo Easy column, packed with 3.0 µm C18 beads. The column was heated at 30 ^{0}C in a Thermo Easy spray oven and the peptides were separated using linear gradient from 5% to 35% of buffer B (95% acetonitrile

and 0.5% acetic acid) at a flow rate of 300 nl/min, followed by a wash reaching 95% of buffer B. Gradient length was adjusted to 140 minutes.

The Dionex UPLC system was directly connected with Thermo Fisher Scientific Q Exactive Orbitrap instrument using Thermo Easy spray source. The Easy source was operated with 2.2 KV and the ion transfer tube at 200 ^{0}C without sheath gas. The mass spectrometer was acquired in data dependent mode. The scans were acquired with a resolution of 70,000 at m/z 400 in Orbitrap mass analyzer with a lock mass of 445.120024 ions. The top 10 most intense peaks containing doubly and higher charged states were selected for sequencing and fragmentation in the HCD mode at a scan resolution of 17,000 with normalized collision energy of 27%, activation time of 10 ms and one micro scan. Dynamic exclusion was activated to minimize repeated sequencing for the entire sequencing event. The peaks selected for fragmentation more than once within 30 s were excluded from selection for next 90 s and the maximum number of excluded peaks was 500.

3.3.7. Data processing and analysis

The acquired raw data were processed using Maxquant. The database search was performed in maxquant with the Andromeda search engine (Thakur et al., 2011) against *Arabidopsis thaliana* database. Parameters used for database search includes trypsin as a specified protease and a maximum of two missed cleavages were allowed. The search included cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modification. Precursor ion mass error tolerance of 20 ppm and fragment ion mass error tolerance of 0.5 Da were allowed. The identification has been done keeping 1% false discovery rate at the peptide and protein level.

3.3.8. Functional annotation and protein-protein interaction networks

Venn diagrams of identified proteins were drawn by using online software Venny 2.1 (Oliveros, 2016). Classification of identified proteins was done by using PANTHER (Protein Analysis Through Evolutionary Relationship) in order to facilitate high throughput analysis. Proteins have been classified according to family and subfamily, molecular function, biological process, cellular components and pathways (Mi et al., 2013). The known and predicted protein-protein interactions for the differentially regulated protein sets were retrieved from STRING v.10 (Search Tool for the Retrieval of Interacting Genes/protein) (Szklarczyk et al., 2015). The protein interaction information was determined from the orthologous proteins of *A. thaliana*. Only those protein interactions with a high confidence

score (≥0.9) were retained in the network with database and experiment as active interaction sources.

3.3.9. RNA extraction and qRT-PCR

Total RNA was extracted from sunflower (both control and inoculated) leaves by using TRI Reagent (Sigma-Aldrich, USA) as per manufactures instructions. RNA yield and purity was estimated by measuring the sample optical density at 260 and 280 nm by using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). First strand cDNA was synthesised by utilizing 500 ng of total RNA by using Super Script III first-strand synthesis kit (Invitrogen, USA) according to the manufactures protocol. The resultant cDNA was used as a template for qRT-PCR. All the primers were designed with Primer-Blast software and list of the genes, forward and reverse primer sequences and their amplicon sizes were mentioned in the table 3.6. Quantitative real time-PCR was performed using an Applied Biosystem ViiA 7 Real-Time PCR system (Applied Biosystems). Each 10 µl PCR reaction volume contained 1 µl of c-DNA; 0.5 µl of each forward and reverse primer (8 pM); 3 µl of MilliQ water and 5 µl of Power SYBR Green PCR master mix (Applied Biosystems). The following thermal cycling conditions were used for all reactions: 50 ^{0}C for 2 min; 95 ^{0}C for 10 min; 40 cycles of 95 ^{0}C for 15 s; 60 ^{0}C for 30 s and 72 ^{0}C for 15 s. The sunflower Actin gene was used as an endogenous control, and relative fold change of the various gene transcripts was calculated according to the deta-delta method; relative expression= $2^{-(\Delta Ct\ sample - \Delta Ct\ control)}$.

Table 3.6. Primers for quantitative Real Time-PCR analysis

Gene name	Forward primer Sequence (5'->3')	Reverse primer Sequence (5'->3')	Amplicon Size (bp)
6-phosphogluconate dehydrogenase	ATGCGGGGTTGGAAGAAGAG	ACTCTCTTCGCCCTCAACAA	142
Formate dehydrogenase	ACTCCATTCCACCCTGGCTA	GCAACAGTTAACCCAGCGTC	134
Catalase	ACACTCAGAGGCACCGTCTT	GCTTTCCGGACAACCTAACA	201
HSP	CGTCGTCTCCATCAAGGTTT	CTGCTTCCCTAACCATACGC	260
Heat shock transcription factor	CGACTTGCTTCCGACTCTCTT	CCTTGTTGGCCCATGATTTCC	179
Pyruvate dehydrogenase	TTACTAGTGATTCGAGCGGC	AAGCGGCTATTCGAAGCGAT	198
ATP-sulfurylase 3	CAACCGACCCGTCTCCTAAC	ATTCGATTAGCGTGGTCGC	199
LHCP	AGCTGGAGGTGATCCACTGC	CCCATCAGGATCACCTGGGTG	220
Glutamine synthetase	GACCTCTCTGATTCCACCCC	CTGTCTTCTCCAGGGGCTTG	173
Actin	AGCTGCTGGTATTCACGAGACC	TCGATCCTCCGATCCAGACACTG	224

3.4. Inheritance and molecular mapping of powdery mildew resistance genes in sunflower (*Helianthus annuus* L.)

3.4.1. Plant material

A population of 264 F_2 individuals was produced from the cross between the highly susceptible accession PS 2023 and resistant accession TX 16R and scored for powdery mildew incidence (phenotyping). These populations were further advanced for development of recombinant inbred F_6 lines. A population of 93 F_6 -derived RILs was analyzed for powdery mildew resistance with regard to phenotyping and genotyping. Both F_2 and RIL population were used for inheritance studies.

3.4.2. Powdery mildew disease assessment

The powdery mildew responses were recorded in both F_2 and RILs at natural infection in field conditions. Both field trials were conducted at the field farms of IIOR, Hyderabad. The highly susceptible genotype PS 2023 was used as infector row at every 10th row of the plot. Each F_6 derived recombinant inbred line was sown in a row design with 10-15 plants per row. Disease score was recorded at maturity stage of plants. The disease reaction was assessed by using the 0-9 scale (0= immune, 1=highly resistant, 2=resistant, 3-4=moderately resistant, 5-9=susceptible/highly susceptible) based on an infection percentage on leaf and observed on the whole plant or confined till the middle leaves or only to the lower leaves.

3.4.3. DNA extraction

Genomic DNA was isolated from young leaves of parents and their RILs with CTAB method with minor modifications (Doyle and Doyle, 1990). The plant material was ground in liquid nitrogen, then homogenized in 20 ml of extraction buffer (2% CTAB, 20 mM EDTA, 2% PVP, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0 and 1% β-mercaptoethanol) and incubated at 65 ^{0}C for 1 h. The supernatant was twice extracted with chloroform: isoamylalcohol (24:1 v/v) and treated with RNase A (100 μg/ml), incubated at 37 ^{0}C for 30 min. The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and resuspended in 500 μl of sterile MilliQ water and stored at –20 ^{0}C. The DNA concentration was determined electrophoretically using known amount of λ DNA as standard.

3.4.4. Simple sequence repeat analysis

A total of 484 SSR primers were used to assess the parental polymorphism and 64 polymorphic primers (Table 3.7) that were distributed evenly in all the 17 linkage groups of sunflower were used in linkage map construction and QTL analysis. PCR reaction mixture contained 7.5 mM dNTPs, 3.75 pM primers each of the forward and reverse primer, 1 U *Taq* DNA polymerase, 1X PCR buffer with 1.5 mM $MgCl_2$ (Bangalore Genei, India), and 50 ng of template DNA and sterile distilled water to a final volume of 25 µl. PCR amplification was carried out in a thermal cycler (Applied Biosystems GeneAmp 9700) using the following amplification conditions: 5 min at 94 ^{0}C for the initial denaturation, followed by 45 cycles consisting of 40 s of denaturation at 94 ^{0}C, 1 min of annealing at 62 ^{0}C (varied from primer to primer), and 1.5 min of extension at 72 ^{0}C with a final extension at 72 ^{0}C for 5 min. The PCR amplified products were resolved by electrophoresis on 3.0% agarose (Bangalore Genei, India) gel in 1 X Tris-acetate-EDTA buffer by electrophoresis at 100 V for 3 h and visualized with ethidium bromide staining. The gel images were recorded using the Alpha Innotech Fluorchem gel documentation system.

Table 3.7. List of polymorphic primers used in genotyping the RIL population derived from the cross between PS 2023 and TX 16R

Marker name	Sequence name	Repeat motif and length	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Reference allele length (bp)	Annealing temperature (^{0}C)
ORS 371	EG0105	(GT)7	CACACCACCAAACATCAACC	GGTGCCTTCTCTTCCTTGTG	254	58
ORS 474	EG0308	(AC)14	TGCACCTTTGTTTGGATCTTC	GTGCTCGGGATTGATTCTGT	259	58
ORS 610	EG0536	(AG)14	TTGTGACCTTCTCCCTGCTC	AGGAAGCGAAACGAGGAAGT	144	58
ORS 716	EG0689	(AG)24	CCCCACAACCCATAGCCTAA	GAACTAACCGCCATCCAAGA	312	58
ORS 965	EG1310	(CTAT)34(AT)6(GT)10	TTGGATTACCTTGGATAGTCAGC	CTTACCCTCCTCAGACCCTACCT	362	59
ORS 986	EG1368	(GT)9	GGCTTATGCGACTTGTGTGAAT	CTTGAAACATATCCGTTCCGTTC	291	59
ORS 342	EG0060	(GT)10	TGTTCATCAGGTTTGTCTCCA	CACCAGCATAGCCATTCAAA	342	58
ORS 925	EG1186	(AC)16	ATGATTCTAAGTTGCGGTAGTGC	GTTGGGTTTAAGTTGTTGCTTCC	201	59
ORS 229	2C4	(CAA)12	TCCGACCCGAATCTTATGAACC	GACCCGAATGAGACCCAAACTG	176	51
ORS 338	EG0053	(GT)6(AT)4(GT)12(AT)4(GT)20	CGTGTCAATTAGGTTACGAGTTTG	GTTAGCGAGCCCTAGCAAGA	183	58
ORS 432	EG0219	(AC)11	TGGACCAGTCGTAATCTTTGC	AAACGCATGCAAATGAGGAT	163	58
ORS 1114	EG1647	(CT)13	AGATGGTGGCAGGAGAGTTAAAG	GCAGAAACAGATCAGGAGGGTAT	253	59
ORS 681	EG0637	(CT)15	TGAGCCCTTCGAGTTCTAATTG	ATCGCACCACTCTACCAAGG	391	58
ORS 1197	EG1851	(CT)16	CCCAGTACGTTACAGTCGTGTGTT	CAAACAATCACGCAAGGGTTTA	209	59
ORS 1288	EGB593	(CT)23	TGTCACACTTCCTTCATCAACC	ACAGTGATAATGGTGGTCACTCC	324	52
ORS 538	EG0438	(ACC)8	CTTAACACTTGGTTTGTTGTGTCA	GGGAAGGAGTTAGTGGTGGA	442	58
ORS 546	EG0449	(CT)17	CATGAACATCGCCAATTCAG	TGCAAGGAACCATCAGAATC	205	58
ORS 1028	EG1470	(AG)8	CTTATTCCAAGGACGCATAGTCG	CGATGGTATGATTCTCGACGTTA	207	59
ORS 483	EG0325	(GT)15	CCGAACAACAATCTCCACAA	GGTTTAGGTGTCGCATCACA	271	58
ORS 678	EG0634	(AG)14	TTGCTGTCTCTCCCATAACTGA	GAAAGCGCCCATACACACAT	178	58
ORS 1229	EG1921	(GT)8(GA)7	GAAACAGAGTGAGGCAAACTTCA	CAGCGCCGTATCGTATAGAGTAA	343	59
ORS 700	EG0667	(AG)8	GTACCCACCACGCTTAACCA	AGTCTTCCACAGCAACGTCA	339	58
ORS 702	EG0669	(AG)7	CACGTCATACGATCATGCAA	GACCCATTAGGCAACCGAGT	369	59

ORS 928	EG1200	(GTGC)8(GT)14	CATGGTTATTTTGGTTTGGGTTT	GCTATTATCATGTCCTTGTCCTTTT	214	55
ORS 826	EG0891	(GT)8	TTAATCCATTGTTAGCGTCCTCA	GTAAGGCATTCAACCCACATGAC	146	59
ORS 894	EG1115	(GT)14	TTTCCTCATGATCCCGATTCTAT	TGCATTACCTAATTTCTAGTGGGTTT	252	59
ORS 1013	EG1432	(CT)14	GTCACCAAGTCCCTGTCCTAAA	TGTGTCGGATGAAAGAAGAAATG	184	59
ORS 442	EG0238	(GT)8(GA)7	TGGGTGTTTCCCATTTGAATA	ACGTAGTGCATAAAAGATAACCAGAA	395	58
ORS 510	EG0396	(GT)11	CATCGCGTCCCTCTCTCTAA	CCAACCATCACAGCAATCAG	255	58
ORS 844	EG0947	(AC)17	ACGATGCAAAGAATATACTGCAC	CATGTTTAATAGGTTTTAATTCTAGGG	307	59
ORS 1265	EG2010	(CT)18	GGGTTTAGCAAATAATAGGCACA	ACCCTTGGAGTTTAGGGATCA	222	59
ORS 78	ORS-78	(AAG)10	GTT CGT CGA GTA CAT GTT CTG C	TTT CCC TCT GGA AAG TTG TCA	161	58
ORS 541	EG0441	(AG)17	TGAATTCCTTTTCATTTCAATCAG	ACAGCCACAGGAGAAGGTTG	295	58
ORS 691	EG0651	(CT)19	GCATCTGAGCAACTGCGTTA	ACCGTCCTTAGCTCTTGTGAG	447	58
ORS 815	EG0843	(CTT)8	GGAAAGCAGCAATGGTTCATAA	CACCAAGTGCAAACCCTAGAAA	179	59
ORS 853	EG0986	(AC)12(ACAT)10	TTGTTAAGGCTCTTGCTAATGAA	TGCTTATTGACGTTACCCATTG	230	59
ORS 889	EG1106	(GT)15	ATCAACTACGTCACGATACTCC	GTTCTCATGGATTCTCACAACTC	138	59
ORS 1008	EG1425	(AG)5	GATCACCTTCACTATCCACAACC	CATGAGGGCATTCTTGTCATTT	304	59
ORS 1088	EG1596	(CT)15	ACTATCGAACCTCCCTCCAAAC	GGATTTCTTTCATCTTTGTGGTG	252	59
ORS 1110	EG1634	(CT)10	CATTCAAGGGGCTATTGTGTAAG	GGTTTTGGAGAGGTCGATGTG	297	59
ORS 5	ORS-5	$(aac)_4$	ATG TGG AGC AGC AAA TTC AG	CTG CTG CCC ACC ATA CTG	311	55
ORS 1091	EG1601	(CT)16	TCCTTTAGCCTCACACGAAATC	ACCGTTTACCCATATTACACCTTT	378	59
ORS 1146	EG1708	(AG)15	GGCTCATCACTTGCATCTATTGT	TGAAGACACCATCTCCAATGC	379	59
ORS 502	EG0373	(AACAC)7	ATCCCAACAGACGCCATTAT	AACATTGGAGGGAGCCAATA	120	58
ORS 810	EG0833	(CT)20	TTCAACGTGCGTGATTAAGG	GCGATTTCTTTGGAGACGAA	398	59
ORS 946	EG1243	(CT)16(CA)12	CAAAACCAGCCATCTATCTCTCTTC	TTTGTGGGTCTCTCTCTACAAGGTT	191	59
ORS 707	EG0676	(CT)21	GCAGTCAATTCGTAGCATCG	GCTGAAGCTGAAGACAGATCC	160	60
ORS 1030	EG1474	(GGT)5	CCTTTGATGTAGTTAAGGAAGTTGTG	CGATCAATTTATATGACCGAATTACC	430	59
ORS 578	EG0489	(CT)21	CTCTCAATCCCTAAAGTCCCCT	TGGTGGATGTGGTTGTTGAT	238	58
ORS 832	EG0905	(GT)9	GTGACATTTTCGGACATCATTATT	TCTCTCTATAACACTCGCTCACACA	354	59

ORS 1086	EG1594	(CT)11	TTGTTTGTCGCACACTCAAGATT	ATTATCGGCACATCTTTGGATTT	140	59
ORS 148	5H9	(AC)14	ATCCGTATATTGCCTTGTTGAG	AGAAGCCACGCAATTGATTATC	256	52
ORS 687	EG0647	(CT)14	ACCGTTACACTTATTGGTTATTTCATT	GGGGTTTGTTGTTCTGTTTTG	168	58
ORS 1271	EG2017	(CT)19	GGAATGATTTAAACACCCCTCAA	GTCGGTATGAATGCTGTAAACCC	210	59
ORS 420	EG0194	(GT)13	TCATGGTGTTTGGTTTGTGTC	TGCCAAATTCCTCTTCTTTCT	138	54
ORS 126	1C10	(GT)22	CACTGTCCCTTCTGGTAGTTCC	TTCCCACGCAAACTTCAATTCC	293	51
ORS 310	EG0018	(AC)27	AATTCCCACGCAAACTTCAA	GGGTAAATGGGGCAACCTAT	189	58
ORS 1064	EG1556	(CT)19	TGAATGATCTATGAGTGGTGATGG	ACTCGCAGTGGTAAGTCGTTAGG	245	59
ORS 169	8F10-A	(CA)8 N34 (CA)8	TGGAACTGTAAATGGACCCAAG	GCACTGCACCATTTATGAGAAG	198	55
ORS 170	8F10-B	(TC)7	GCAGTGCCACTCATTTCTGTTG	TGTGATGATTGCAGGATTGGAG	116	52
ORS 513	EG0402	(AC)13(A)39(G)11	AGTCAAATCGCTGCCACCTC	ATTGTCACTGGCTTCCTTGG	228	60
ORS 1097	EG1613	(CT)11	GACCAAGTGGACTGACACGAG	CGGTGGTGGCTTAGATTGTATAGT	161	59
ORS 370	EG0104	(AC)13	CGCGAGTTACGCTTACTGTG	TATGCAATGGACGTGGACAT	295	52
ORS 244	16D5	(GGC)5	AGGTGAATCAACGAGTGAATGG	CACCACCACCGCCGTCTC	233	54

3.4.5. Construction of molecular linkage map

A linkage map was constructed by using QTL IciMapping software (Meng et al. 2015). The map distances were calculated based on Kosambi's function (Kosambi, 1944). Data generated after genotyping of 93 RILs by polymorphic SSR markers were tested using the $\chi 2$ goodness of fit test for a 1:1 segregation ratio.

3.4.6. QTL mapping

QTL mapping was performed with single marker analysis, interval mapping and inclusive composite interval mapping function of QTL IciMapping software v3.3 (http://www.isbreeding.net/software/?type=detail&id=14). Single marker analysis served as the primary method of detecting association between markers and the target traits. Two or more closely linked markers that showed significant association were assumed to identify the same QTL. To determine the precise location of the putative QTLs, interval mapping and composite interval mapping functions were used. Inclusive Composite Interval Mapping (ICIM) (Wang, 2009) was used to confirm QTLs and estimate the phenotypic variation explained (PVE), as implemented in the integrated software QTL IciMapping for building genetic linkage maps and mapping quantitative trait genes (available from http://www.isbreeding.net).

Chapter 4

Development of a scoring scale for powdery mildew (*G. orontii/* formerly *G. cichoracearum/* formerly *Erysiphe cichoracearum*) disease and identification of resistance sources in cultivated and wild sunflowers

The confirmation of the genus causing powdery mildew was undertaken. Differential methods of artificial screening were tested and a new scale for disease scoring has been developed. We have identified new sources of resistance in wild and cultivar germplasm under natural conditions followed by their confirmation through artificial assays and results and discussion were presented in the following chapter.

4.1. Results

4.1.1. Confirmation of the pathogen

The microscopic examination (Fig. 4.1a) of the fungus showed erect conidiophores with chains of cylindrical conidia. The basal septum of the conidiophores was adjacent to the mycelium and there were no fibrosin bodies, which are typical morphological features of *G. orontii*. The universal powdery mildew primer pair failed to gave specific bands; and it always gave a non-specific amplicon, which could be used for detecting the specific genus (Chen et al., 2008). A PCR product of 391 bp (Fig. 4.1b) was amplified with *G. orontii* specific ITS primer pair, while there were no PCR amplification products with *Leveillula taurica* and *Podosphaera xanthii* specific ITS primers. Subsequently, pathogen samples obtained from resistant and susceptible cultivars including the wild species and also from other regions in Southern India were also subjected to microscopic and molecular analysis, which confirmed that the pathogen causing powdery mildew in sunflower in Southern India is *G. orontii*. The amplicon was sequenced and similarity blast results in 98 % homogeneity with *G. orontii* (Fig. 4.1c)

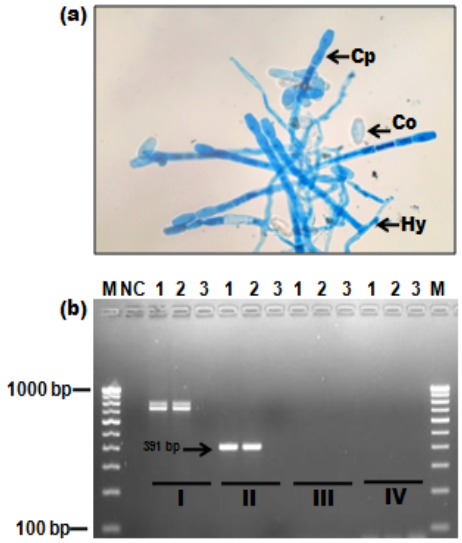

Fig. 4.1 (a) Light microscopy of *G. orontii* (lacto phenol cotton blue stained) with conidiophores (*Cp*), conidia (*Co*) and hyphae (*Hy*). **(b)** PCR confirmation of *G. orontii* infection using ITS primers (M – 100 bp marker, NC - negative control, 1 - powdery mildew fungi, 2 - sunflower leaf infected with powdery mildew, 3 - sunflower leaf free of infection and I - amplification with ITS primers specific for all powdery mildew fungi, II - amplification with ITS primers specific to *G. orontii,* III - amplification with ITS primers specific to *Leveillula taurica,* IV - amplification with ITS primers specific to *Podosphaera xanthii.*

Secure | https://blast.ncbi.nlm.nih.gov/Blast.cgi#822094469

Download GenBank Graphics

Golovinomyces orontii isolate gc-s-hyd 18S ribosomal RNA gene, partial sequence; transcribed spacer 2, partial sequence

Sequence ID: KP834315.1 Length: 416 Number of Matches: 1

See 1 more title(s)

Range 1: 1 to 386 GenBank Graphics Next Match Prev

Score	Expect	Identities	Gaps	Strand
680 bits(368)	0.0	383/389(98%)	5/389(1%)	Plus/Minus

```
Query  8    GAGGGGGTGTG-CGACGCTCG-ACAGGCATGCCCCTCGGAATGCCAAGGGGCGCAATGTG  65
Sbjct  386  GAGGGGGTGTGACGACGCTCGAACAGGCATGCCCCTCGGAATGCCAAGGGGCGCAATGTG  327
Query  66   CGTTCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACGTATCGCATTTCGCT  125
Sbjct  326  CGTTCAAAGATTCGATGATTCACTAAATTCTGCAATTCACATTACGTATCGCATTTCGCT  267
Query  126  GCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTAACAATTCAAAT  185
Sbjct  266  GCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGAAAGTTTTAACAATTCAAAT  207
Query  186  AGTTTTCCTCAGACTACACGACAACACGAGTTAGGTTGGGTCTTTGGCGGACACGAGCCA  245
Sbjct  206  AGTTTTCCTCAGACTACACGACAACACGAGTTAGGTTGGGTCTTTGGCGGACACGAGCCA  147
Query  246  GCACAGAGCCGGCCGGCGCGCGAGGCACCTGGCCCGCCAAAGCAACATGAGATAAGTCAA  305
Sbjct  146  GCACAGAGCCGGCCGGCGCGCGAGGCACCTGGCCCGCCAAAGCAACATGAGATAAGTCAA  87
Query  306  CACGGGTGGACGGTCAACTCTGCGCGCGGGGCAAGCCCGGGCAGAGTCTCACGCTCTGTA  365
Sbjct  86   CACGGGTGGACGGTCAACTCTGCGCGCGGGGCAAGCCCGG-CAGA-TCTCACGCTCTGTA  29
Query  366  ATGATCCTTCCGCAGGTCCACCTAACGGA  394
Sbjct  28   ATGATCCTTCCGCAGGTTCACCTA-CGGA  1
```

Fig. 4.1 (c) Similarity blast results of sequence with that of *G.orontii.*

4.1.2. Infection methods for artificial assays

All the seven methods used for artificial infection resulted in infectivity. The spore load (conidia/cm^2) varied from 2,500 to 4,460, 6,000 to 8,600 and 61,100 to 85,990 in ID-25, TX 16R and Morden, respectively (Table 4.1). Differences in the spore load were significant among the genotypes tested and treatment differences within the genotypes with regard to the methods of infection were also significant. Of all the methods, stapling method recorded significantly higher spore load in the three genotypes probably due to overestimate of the spores that dislodge from the stapled infected leaf. However, stapling and blotter paper methods caused mechanical damage and leaf vitrification; respectively. Dipping the leaves in spore suspension, swabbing with cotton wool, spraying the spore suspension and touching the leaves of test plants with infected leaves resulted in delayed sporulation even in the susceptible lines. Dusting of powdery mildew conidia on healthy leaves resulted in uniform and good levels of infection without any tissue damage. Hence, the method of conidial dusting from infected leaves was employed for artificial screening of resistant material due to convenience in operation and also the level of infection obtained.

Table 4.1 Spore load on resistant and susceptible genotypes with different artificial infection methods

Infection method	ID-25 (Resistant) Conidia/cm^2	TX 16R (Resistant) Conidia/cm^2	Morden (Susceptible) Conidia/cm^2
Dusting conidia on test plant	4,067[a]	7,067[b]	67,500[e]
Spraying the spore suspension	3,067[c]	7,000[bc]	65,280[f]
Swabbing with cotton wool	3,233[bc]	6,700[c]	61,100[h]
Dipping the test leaves in spore suspension	2,500[d]	6,000[d]	70,870[d]
Stapling method	4,460[a]	8,600[a]	85,990[a]
Blotter paper method	2,870[cd]	7,000[bc]	76,400[b]
Rubbing the healthy leaves with infected leaves	2,600[d]	7,000[bc]	62,400[g]

Means in a column followed by same letters are not significantly different according to DMRT at $P < 0.001$

4.1.3. Disease assessment

As the material was from diverse genetic backgrounds exhibiting significant variations in qualitative and quantitative characters, infection was observed on the whole plant or confined till the middle leaves or only to the lower leaves (Fig. 4.2). Hence, a scale was developed based on the level of infection on the bottom, middle and top leaves (Table 4.2).

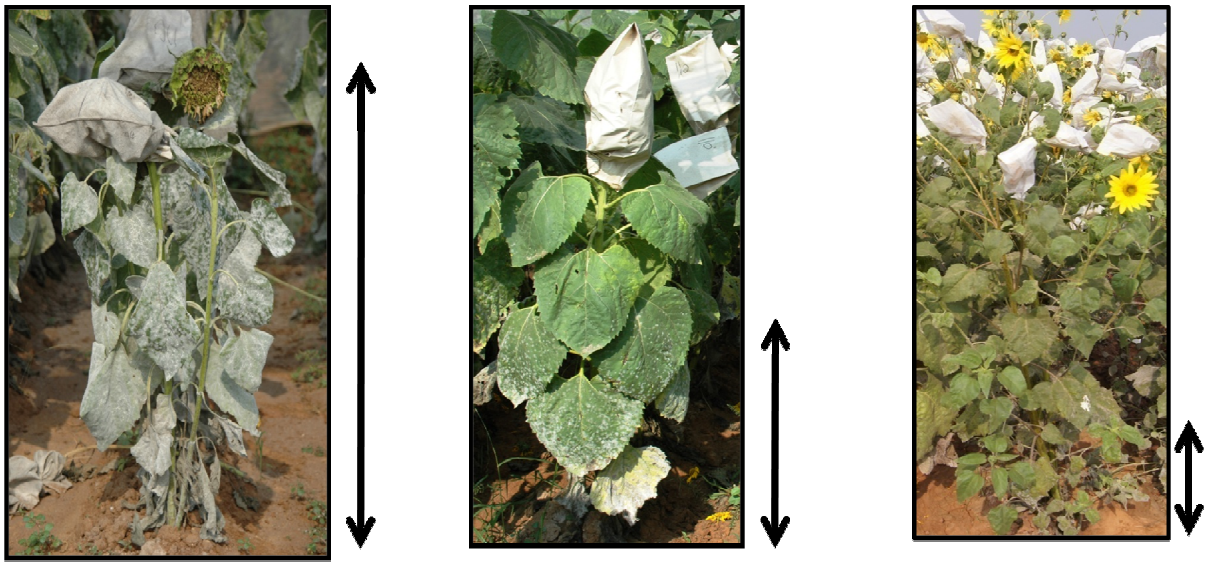

Fig. 4.2 Infection was observed on the whole plant or confined till the middle leaves or only to the lower leaves.

Table 4.2 0-9 scale for scoring powdery mildew incidence

Position of leaf	Bottom	Middle	Top	Scale	Category
Severity of powdery mildew	High	High	High	9	Highly susceptible
	High	High	Medium	8	Highly susceptible
	High	High	Low	7	Susceptible
	High	Medium	Medium	6	Susceptible
	High	Medium	Low	5	Susceptible
	High	Low	Low	3	Moderately resistant
	Medium	Medium	Medium	4	Moderately resistant
	Medium	Medium	Low	4	Moderately resistant
	Medium	Low	Low	2	Resistant
	Low	Low	Low	1	Highly resistant
	0	0	0	0	Immune

0 = no infection, Low = < 10% infection, Medium = 11-30% infection, High = 31-100% infection

4.1.4. Reaction of *Helianthus* species to *G. orontii*

Table 4.3 shows the reaction of wild *Helianthus* accessions to *G. oronttii* under field and greenhouse conditions. The wild sunflower species showed variability in their reaction to powdery mildew ranging from immune to highly susceptible reaction. Most of the accessions showed the same type of reaction under both field and artificial inoculation conditions. However the accessions, EGG-1629, HIR-03, MOL-1530, GRA-2043 that were immune and OCC-52, NUT-1517, TUB-15, STR-1934, STR-216 (moderately resistant) under field evaluation, were found susceptible under artificial infection. The accessions PAU-S-869, RES-09 and TUB-1705 that were found to be immune under field evaluation showed highly resistant reaction under artificial conditions with very low infection. The wild species, *H. argophyllus*, *H. angustifolius, H. agrestis, H. atrorubens, H. debilis*, *H. rigidus*, *H. salicifolius* and *H. praecox* were found to be immune (Fig. 4.3). The accessions of *H. pauciflorus* and *H. resinosus* were highly resistant, *H. laevigatus*, showed moderate resistance. The accessions of *H. bolanderi*, *H. divaricatus*, *H. eggertii*, *H. hirsutus*, *H. microcephalus*, *H. mollis*, *H. occidentalis*, and *H. petiolaris* subsp. *petiolaris* were susceptible under both natural and artificial inoculation conditions. Intra-accessional variability was found in some species such as, *H. decapetalus*, *H. giganteus*, *H. grosseserratus*, *H. maximiliani*, *H. nuttallii*, *H. strumosus* and *H. tuberosus.* Though the disease index among some of the wild species was similar, differences were observed in the spore load (Table 4.3). The Duncan's Multiple Range Test showed significant differences in the spore load levels among the species. Correlation between disease index under artificial conditions and spore load was positive and highly significant (r=+0.90496).

Fig. 4.3 Reaction of wild Helianthus species to powdery mildew (Field evaluation), a) *H. salicifolius* and *H. strumosus*, b) *H. maximiliani* and *H. resinosus* c) *H. debilis*, d) *H. decapetalus*, e) *H. strumosus*, and f) *H. praecox*.

Table 4.3 Reaction of *Helianthus* species to powdery mildew under natural and artificial infection conditions

Species	Accession no	PI number	Plant habit	Ploidy	Disease index		Spore count (no of conidia/cm^2)
					Natural infection (30 plants)	Artificial infection (30 plants)	
H. agrestis Pollard	AGR-1567	PI 468414	A	D	0	0	0^{s}
H. argophyllus	ARG-19	-	A	D	0	0	0^{s}
H. bolanderi	BOL-1023	PI 649867	A	D	9	9	44,533^{cd}
H. debilis ssp. *debilis* Nutt.	DEB-689	PI 468689	A	D	0	0	0^{s}
	DEB-692	PI 468692	-	-	0	0	0^{s}
H. petiolaris ssp. *petiolaris* Nutt.	PET-550	PI 435825	A	D	9	9	58,900^{a}
H. praecox ssp. *praecox* Engelm. & A. Gray	PRA-1823	PI 494600	A	D	0	0	0^{s}
	PRA-1824	PI 494601	-	-	0	0	0^{s}
H. angustifolius	ANG-1241	PI 468419	P	D	0	0	0^{s}
H. atrorubens	ATR-1902	PI 503206	P	D	0	0	0^{s}
	ATR-1581	-			0	0	0^{s}
H. decapetalus L.	DEC-01	-	P	D, T	5	7	34,667^{efg}
	DEC-1887	-	-	-	9	9	48,500^{b}
	DEC-1922	-	-	-	0	0	0^{s}
	DEC-2054	-	-	-	0	0	0^{s}
	DEC-970	PI 649970	-	-	3	3	14,533^{p}
H. divaricatus L.	DIV-1881	-	P	D	7	9	26,467^{ijk}
	DIV-1885	-	-	-	7	9	20,233^{mno}
H. eggertii Small.	EGG-1629	-	P	H	0	5	23,500^{klm}
H. giganteus L.	GIG-184	PI 547184	P	D	0	1	4,133^{qrs}
	GIG-2014	-	-	-	8	9	56,800^{a}
	GIG-2015	-	-	-	7	7	22,933^{klm}
	GIG-2017	-	-	-	7	9	40,667^{cd}
	GIG-984	PI 649984	-	-	9	9	38,733^{de}
H. grosseserratus Mar.	GRA-10	-	P	D	7	8	30,767^{ghi}
	GRA-2043	-	-	-	0	9	41,400^{cd}
	GRA-2212	-	-	-	3	3	3,733^{rs}
	GRA-977	-	-	-	9	9	25,367^{jkl}

H. hirsutus Raf.	HIR-03	-	P	T	0	5	17,033^{op}
	HIR-1536	-	-	-	7	9	32,067^{fgh}
H. laevigatus T. & G.	LAE-5709	PI 435709	P	T	3	4	8,647^{q}
H. maximiliani Schr.	MAX-007	PI 650007	P	D	3	3	6,667^{qr}
	MAX-010	PI 650010	-	-	9	9	23,600^{klm}
	MAX-11	PI 650011	-	-	3	3	6,700^{qr}
	MAX-1631	-	-	-	7	9	31,500^{gh}
	MAX-2010	-	-	-	8	8	29,100^{hij}
	MAX-30	-	-	-	9	9	21,166^{lmno}
	MAX-33001	-	-	-	9	9	22,867^{klm}
H. microcephalus T.&G.	MIC-1872	PI 503231	P	D	9	9	44,267^{c}
H. mollis Lam.	MOL-1530	-	P	D	0	5	22,233^{klmn}
	MOL-1629	-	-	-	7	7	30,700^{ghi}
H. nuttallii ssp. *nuttallii* T. & G.	NUT-023	PI 650023	P	D	0	0	0^{s}
	NUT-05	-	-	-	5	7	23,267^{klm}
	NUT-1517	-	-	-	3	8	31,800^{gh}
H. occidentalis ssp. *occidentalis* Riddell.	OCC-52	-	P	D	3	9	55,500^{a}
H. pauciflorus	PAU-S-869	PI 435869	P	H	0	1	2,900^{rs}
H. resinosus Small.	RES-09	-	P	H	0	1	4,100^{qrs}
H. rigidus	RIG-1696	-	P	H	0	0	0^{s}
H. salicifolius Dietr.	SAL-241	PI 435872	P	D	0	0	0^{s}
H. strumosus L.	STR-15	-	P	D, H	9	9	36,233^{ef}
	STR-1934	-	-	-	3	9	37,667^{de}
	STR-216	PI 547216	-	-	3	5	18,100^{nop}
	STR-251	PI 503251	-	-	1	1	4,100^{qrs}
H. tuberosus L.	TUB-05	-	P	H	7	9	32,133^{fgh}
	TUB-07	-	-	-	7	7	28,700^{hij}
	TUB-15	-	-	-	3	9	49,033^{a}
	TUB-1705	-	-	-	0	1	3,733^{rs}
	TUB-2069	PI 547243	-	-	9	9	30,933^{gh}
	TUB-2729	AMES2729	-	-	7	7	32,900^{fgh}

Ploidy: D = diploid (n = 17), T = tetraploid (n = 34), H = hexaploid (n = 51). Growth habit: A= annual, P = perennial. Means in a column followed by same letters are not significantly different according to DMRT at P < 0.001.

4.1.5. Reaction of prebred lines

Among 195 prebred lines, 21 accessions showed moderate resistance while the remaining accessions were found to be susceptible to the disease. None of the lines were immune or highly resistant (Table 4.4). The mean disease index ranged from 22 to 100 with an average of 68. The AUDPC ranged from 144 to 648 with a mean of 445. The days to 50% flowering ranged from 48 to 76 with a mean of 59. There was a positive Pearson correlation between DSI and AUDPC (r = 0.99) and a negative correlation of DSI and AUDPC with days to 50% flowering (r = -0.39, r = -0.39, respectively) (Table 4.6).

4.1.6. Reaction of core germplasm

Out of 112 accessions of the core germplasm, 7 accessions were moderately resistant while all other accessions were found to be susceptible (Table 4.4). None of the lines of the core germplasm were either immune or highly resistant to powdery mildew. The DSI ranged from 33 to 98 with a mean of 81. The mean AUDPC was recorded as 538 ranging from 144 to 648. The mean days to 50% flowering were 61 and ranged from 48 to 83 days while the mean leaf area was 2853 ranging from 166 to 11310. Pearson correlation coefficients were positive between DSI and AUDPC (r = 0.46), DSI and LA (r = 0.23), AUDPC and LA (r = 0.01) while there was negative correlation between AUDPC and days to 50% flowering (r = -0.01) and a weak positive correlation between DSI and days to 50% flowering (r = 0.07) (Table 4.6).

4.1.7. Reaction of exotic lines

A set of 19 exotic lines were evaluated for field resistance to *G. orontii*. Two accessions, TX 16R and EC-537925 (USDA-25) showed resistance (Fig. 4.4) (Table 4.4), five lines showed moderate resistance and the remaining lines were susceptible. The mean DSI ranged from 15 to 100 with a mean of 48 and mean AUDPC ranged from 93 to 648 with a mean of 310. The mean days to 50% flowering were 64 ranging from 54 to 73. There was a positive Pearson correlation between DSI and AUDPC (r = 0.99) and a negative correlation between DSI and days to 50% flowering (r = -0.36) (Table 4.6).

Table 4.4 Reaction of prebred lines, core germplasm and exotic lines to *G. orontii* under field (net contained) conditions

Plant material	Number of accessions	Disease reaction	Disease index Scale	No of accessions	DSI		AUDPC	
					Range	Mean	Range	Mean
Prebred lines*	195							
		Immune	0	0	0	0	0	0
		Highly resistant	1	0	0	0	0	0
		Resistant	2	0	0	0	0	0
		Moderately resistant	3-4	21 (PS 1028, 1078, PS 2029, 2037, 2064, 2065, PS 3005, 3008, 3009, 3010, 3011, 3028, PS 4015, 4037, 4050, 4052, PS 5011, 5032, PS 6002, SOC 11, 17)	22-33	27	145-216	175
		Susceptible/highly susceptible	5-9	174	44-100	75	360-648	487
Core germplasm	112							
		Immune	0	0	0	0	0	0
		Highly resistant	1	0	0	0	0	0
		Resistant	2	0	0	0	0	0
		Moderately resistant	3-4	7 (PI 340790, PI 650343, PI 535894, PI 431542, PI 343798, PI 307934, PI 650781)	33-38	35	216-288	236
		Susceptible/highly susceptible	5-9	105	40-98	82	306-648	527
Exotic lines	19							
		Immune	0	0	0	0	0	0
		Highly resistant	1	0	0	0	0	0
		Resistant	2	2 (PI 642072, EC- 537925)	15-22	18	110-144	127
		Moderately resistant	3-4	5 (803-1, EC-687412, EC-53723, EC-53724, EC-53726)	23-27	23	145-155	145
		Susceptible/highly susceptible	5-9	12	44-100	68	334-648	442
Checks		DRSF-108	5-9			86		528
		Morden				93		590
		PS 2023				98		636

DSI = Disease severity index, AUDPC = Area under disease progression curve

*Prebred lines in 1000 series are derived from *H. argophyllus* x cult. sunflower = 61; 2000 series from *H. petiolaris* x cult. sunflower = 32; 3000 series from Wild *H. annuus* x cult. sunflower =14; 4000 series from *H. argophyllus* x Wild *H. annuus* x cult. sunflower – trispecific = 53; 5000 series from cult. sunflower x *H. debilis* = 17; 6000 series from cult. sunflower x *H. maximiliani* = 6; SOC series from cult. sunflower x *H. occidentalis* = 12; total lines = 195.

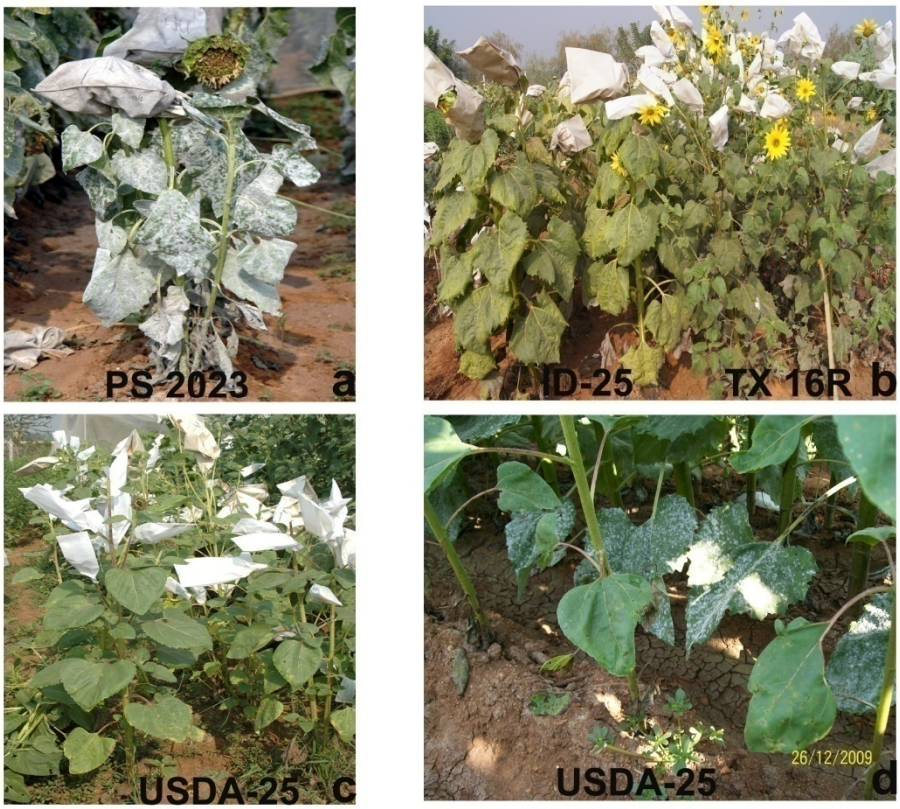

Fig. 4.4 Reaction of identified powdery mildew resistant genotypes to powdery mildew under high infection levels of powdery mildew in field, a) PS 2023 (highly susceptible), b) ID-25 and TX 16R c and d) USDA-25

4.1.8. Reaction of interspecific derivatives

Of the 35 interspecific derivatives that were evaluated for field resistance to *G. orontii* two accessions, HIR-1734-2 and RES-834-3 showed resistance, seven lines showed moderate resistance while the remaining lines were susceptible (Table 4.5). The mean DSI ranged from 17 to 95 with a mean of 57. The mean AUDPC ranged from 97 to 625 with a mean of 369. The mean days to 50% flowering was 65 ranging from 57 to 77 days and the leaf area was 2641 ranging from 632 to 8776. There was a positive Pearson correlation between DSI and AUDPC (r = 0.99) and a negative correlation between DSI with days to 50% flowering (r = -0.36) and LA (r = -0.36); AUDPC and LA (r = -0.37), AUDPC and days to 50% flowering (r = -0.35) (Table 4.6).

Table 4.5 Reaction of interspecific derivatives to *G. oronttii* under field (net contained) conditions

IIOR Identity	Germplasm	Reg. no.	PI Number	Pedigree	DSI	AUDPC
ID-1	ANO-1509-1	GP-135	PI 539894	cmsHA 89*2/*H. anomalus* (Acc. 1509)//RHA 265 F_4	27	185
ID-2	ANO-1509-2	GP-136	PI 539893	cmsHA 89*2/*H. anomalus* (Acc. 1509)//RHA 265 F_4	33	216
ID-3	BOL-774	GP-143	PI 539892	cmsHA 89*2/*H. bolanderi* (Acc. 774) F_3	72	454
ID-4	DEB-CUC-1810	GP-146	PI 539911	nms P21*3/*H. debilis* ssp. *cucumerifolius* (Acc. 1810) F_3	70	447
ID-5	DEB-SIL-367-2	GP-145	PI 539908	cmsHA 89*3/*H. debilis* ssp. *silvestris* (Acc. 367) F_3	52	327
ID-6	DES-1474-1	GP-162	PI 539912	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4	87	576
ID-7	DES-1474-2	GP-163	PI 539913	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4	82	527
ID-8	DES-1474-3	GP-164	PI 539914	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4	83	530
ID-9	GIG-1616-1	GP-237	PI 610782	nmsHA P21/*H. giganteus* (Acc. 1616) BC_1F_2	95	626
ID-10	GIG-1616-2	GP-238	PI 610783	nmsHA P21/*H. giganteus* (Acc. 1616) BC_1F_2	83	526
ID-11	HIR-828-1	GP-239	PI 610784	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	51	337
ID-12	HIR-828-2	GP-240	PI 610785	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	30	178
ID-13	HIR-828-3	GP-241	PI 610786	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	82	527
ID-14	HIR-828-4	GP-242	PI 610787	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	50	317
ID-15	HIR-1734-1	GP-150	PI 539907	cmsHA 89*2/ *H. hirsutus* (Acc. 1734) F_3	84	547
ID-16	HIR-1734-2	GP-151	PI 539906	cmsHA 89*2/ *H. hirsutus* (Acc. 1734) F_3	21	128
ID-17	HIR-1734-3	GP-152	PI 539905	cmsHA 89*2/ *H. hirsutus* (Acc. 1734) F_3	76	464
ID-18	NEG-1255-1	GP-147	PI 539904	nms P21*2/*H. neglectus* (Acc. 1225) F_3	80	527
ID-19	PAR-1084-1	GP-138	PI 539901	cmsHA 89*2/*H. paradoxus* (Acc. 1084) F_4	32	218
ID-20	PAR-1673-1	GP-160	PI 539899	cmsHA 89*2/*H. paradoxus* (Acc. 1673) F_3	32	207
ID-21	PRA-PRA-1142	GP-149	PI 539910	nms P21*2/*H. praecox* ssp.*praecox* (Acc. 1142) F_5	29	211
ID-22	PRA-RUN-417-1	GP-156	PI 539886	cms HA 89*/ *H. praecox* subsp. *runyonii* (Acc. 417) F_3	22	144
ID-23	RES-834-1	GP-153	PI 539897	cms HA 89*2/*H. resinosus* (Acc. 834) F_5	42	272
ID-24	RES-834-2	GP-154	PI 539896	cms HA 89*2/*H. resinosus* (Acc. 834) F_4	42	238
ID-25	RES-834-3	GP-155	PI 539895	cms HA 89*2/*H. resinosus* (Acc. 834) F_5	17	97
ID-26	STR-1622-1	GP-243	PI 610788	nmsHA P21/*H. strumosus* (Acc. 1622) BC_1F_2	85	529
ID-27	STR-1622-2	GP-244	PI 610789	nmsHA P21/*H. strumosus* (Acc. 1622) BC_1F_2	84	563
ID-28	TUB-346	GP-181	PI 564549	cmsHA 89*2/*H. tuberosus* (Acc. 346) F_3	43	238
ID-29	TUB-365	GP-182	PI 564515	cmsHA 89*2/ *H. tuberosus* (Acc. 346) F_3	49	285
ID-30	TUB-825-1	GP-245	PI 610790	nmsHA P21/ *H. tuberosus* (Acc. 825) BC_1F_2	49	301
ID-31	TUB-825-2	GP-246	PI 610791	nmsHA P21/*H. tuberosus* (Acc. 825) BC_1F_2	78	488
ID-32	TUB-1709-1	GP-183	PI 564517	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_3	81	522
ID-33	TUB-1709-2	GP-184	PI 564518	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_4	70	427
ID-34	TUB-1709-3	GP-185	PI 564519	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_3	44	237
ID-35	TUB-1789	GP-186	PI 564520	cmsHA 89*2/nms P21 VR1*1/*H. tuberosus* (Acc. 1789) F_3	37	230

DSI = Disease severity index, AUDPC = Area under disease progression curve

Table 3.6 Correlation coefficients among various characters under field condition on different germplasm sources

Group	Character	Pearson Correlation Coefficients		
		AUDPC	D/F	LA
Core germplasm	DSI	0.46*	0.07	0.23*
	AUDPC	1.0	-0.01	0.01
Prebred lines	DSI	0.99*	-0.39*	ND
	AUDPC	1.0	-0.39*	ND
Exotic lines	DSI	0.99*	-0.36**	ND
	AUDPC	1.0	-0.37**	ND
Interspecific derivatives	DSI	0.99*	-0.36**	-0.36**
	AUDPC	1.0	-0.37**	-0.35**

DSI = Disease severity index, AUDPC = Area under disease progression curve, D/F = Days to 50% flowering, LA = Leaf area, ND = Not documented

*Indicates significance at $P < 0.01$, **Indicates significance at $P < 0.05$

Table 4.7 provides the data on sporulation of *G. orontii* on the resistant, susceptible and highly susceptible accessions under artificial infection conditions. The sporulation load was significantly low in the resistant accessions as compared to the highly susceptible accessions; the highly susceptible accession (PS 2023) had a high spore count on all the leaves. The resistant accessions had significantly low spore load even on the lower leaves, which in most cases was much less than the spore load on the top leaves of the susceptible cultivars. The DMRT showed significant differences in spore load from bottom to top leaves of the plant. Regardless of the category, the spore count was 2-3 fold less in the top leaves as compared to that on the bottom leaves. However, in case of RES-834-3 and PI 642072, the disease failed to spread to the top leaves. This observation emphasizes the importance of considering the progression of the disease on the plant in devising a scale for disease scoring. Hence, the present study gives a broad scale (0-9) for disease scoring, which allowed identification of resistance sources to *G. orontii* based on the leaf area infected as well as the progression of disease on the plant.

Table 4.7 Reaction of highly susceptible and highly resistant accessions to *G. orontii*

Accession	IIOR identity	Reaction to *G. orontii*	Spore count (conidia/cm^2)			DSI	AUDPC
			Bottom leaves	Middle leaves	Top leaves		
Morden	Morden	Highly susceptible	1,22,460^b	67,692^b	53,750^b	93	590
PS 2023	PS 2023	Highly susceptible	1,62,518^a	83,653^a	72,532^a	98	636
PI 331176	SCG-13	Susceptible	93,126^c	95,281^a	30,917^c	46	307
RES-834-3	ID-25	Resistant	36,737^d	11,205^d	5,106^e	17	97
HIR-1734-2	ID-16	Resistant	35,319^d	21,844^{cd}	17,872^d	21	128
PI 642072	TX16R	Resistant	11,914^e	9,503^d	3,120^e	15	104
EC 537925	USDA-25	Resistant	27,234^d	28,652^c	14,59^e	22	144

DSI = Disease severity index, AUDPC = Area under disease progression curve

Means in a column followed by the same letters are not significantly different according to DMRT at $P < 0.001$

4.1.9. Relation between number of stomata and powdery mildew spore load on leaves of *Helianthus* wild species

Number of stomata are counted in all wild species and powdery mildew spore load/cm^2 on leaf was determined. Data were tested for normality where the spore count was non-fiction in distribution and stomatal count was normal in nature. Hence, performed nonlinear statistical techniques to obtain a relation between these variables. The Spearman rank correlation was used to identify whether there was any relation. It was observed that there was no relation (Fig. 4.5). Log transformation for spore count was done and nonlinear regression analysis was performed yielding no relation.

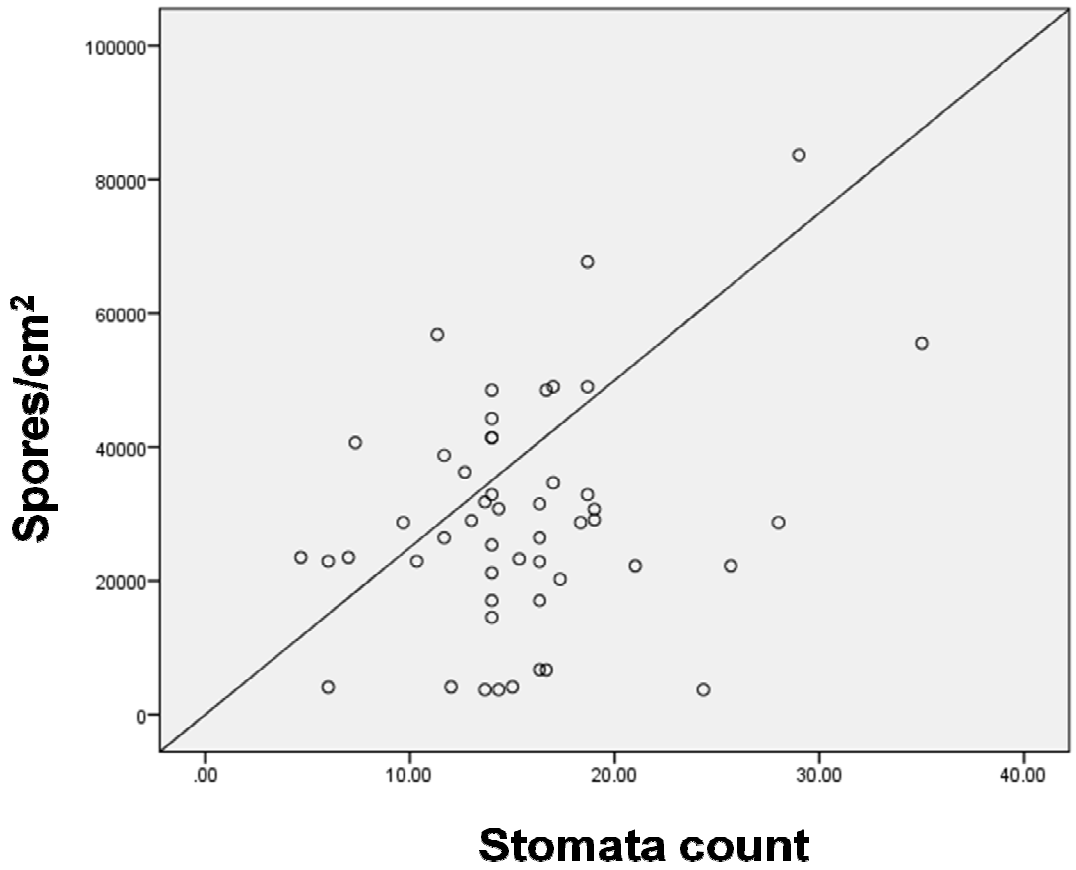

Fig. 4.5 Relations between number of stomata on leaf and powdery mildew spore load

4.2. Discussion

Powdery mildew is a frequently found disease on sunflower in warmer regions of the world (Zimmer and Hoes, 1978). The disease usually appears at post flowering stage when senescence of lower leaves occurs and is reported to cause limited damage in temperate climates. However, the impact of the disease is dramatic in the tropical areas since the powdery mildew instructs the plant to direct energy to the sites of infection (Gulya et al., 1997; http://grdc.com.au). In India, the disease has become serious and occurring regularly for the past decade and is observed during different crop growing seasons and under severe conditions is found infecting the cotyledonary leaves as well. Application of fungicides to manage the disease involves high cost, besides the environmental concern and the insensitivity built up in the pathogen limit their usage (Gullino and Kuijpers, 1994). Hence, there is a need for identifying reliable sources of resistance to powdery mildew. Wild

Helianthus species represent a valuable reservoir of genes for several biotic stresses, which have been successfully introgressed into cultivated sunflower (Seiler 2008). Sources of resistance to powdery mildew have been identified in both annual and perennial *Helianthus* species, but introgression into cultivar background is a long drawn process. Despite the identification of several species as sources of resistance, not much headway has been made in incorporating resistance to powdery mildew as the disease has not assumed economic importance in the temperate regions till date. The present study has been undertaken for screening and identification of potential sources conferring resistance to powdery mildew in the wild *Helianthus* species and cultivar germplasm by developing reliable screening techniques.

Three powdery mildew genera *viz., G. orontii, Podosphaera xanthii (= Sphaerotheca fuliginea)* and *Leveillula taurica* are identified from diseased sunflower leaves of which *G. orontii* is the most commonly reported in all the continents (Fang, 1973; Gulya et al., 1997; Chen et al., 2008). All the three genera are reported from India (Gulya et al., 1997). The symptoms of powdery mildew caused by the three fungi are similar, but these fungi can be distinguished from each other based on morphological characters. Chen et al. (2008) has developed a relatively easy and effective technique based on ITS sequence analysis for reliable detection and differentiation of the powdery mildew genera. The genus causing the powdery mildew disease on sunflower in India is not known. Based on morphological observations and PCR analysis using ITS primers specific to different powdery mildew genera that infect sunflower, it has been confirmed that *G. orontii* is the causative pathogen of powdery mildew in southern India.

Different disease scoring scales were used for estimation of powdery mildew infection in sunflower *viz.,* - a 0-3 scale (Saliman et al., 1982; Dedic et al., 2012), percentage of leaf area infected (Jan and Chandler 1985; Roja-Barros et al., 2004; 2005; Gulya et al., 1991) and a 0-5 scale (McCarter 1993; Dinesh et al., 2010). In the earlier studies, disease evaluation was confined to wild *Helianthus* species and the material was scored as either resistant or susceptible since only 2 disease phenotypes were discernible. In the present study, germplasm from diverse sources was evaluated, which exhibited variable reaction and based on the observations a visual rating of powdery mildew incidence was assigned on a 0 (immune) to 9 (highly susceptible) scale. This allows researchers to have a reliable estimate of the disease based on the percentage of leaf area infected as well as the spread of the disease

on the plant. This scale is particularly important in scoring for the disease in mapping populations where the resistance is polygenically controlled. Based on seven different methods tested in the present study, dusting of healthy plants using leaves infected with *G. orontii* was found useful for artificial assays.

Annual *Helianthus* species exhibited resistance to diseases like rust and downy mildew while perennial species were found to be resistant to *Sclerotinia sclerotiorum* and *Alternaria helianthi* (Gulya et al., 1997). However, resistance in case of powdery mildew is identified in both annual and perennial species (Saliman et al., 1982; Skoric 1984; Jan and Chandler 1985; McCarter 1993; Rojas-Barros et al., 2004; 2006; Christov 2008; Dedic et al., 2012). The Indian Institute of Oilseeds Research maintained perennial *Helianthus* species since 1998 in the field garden and none of the species were reported to harbor the disease until 2007. During 2008, there was a sudden incidence of powdery mildew in cultivar germplasm and also the wild *Helianthus* species. Although the disease was sporadic and confined to spring season in 2008, it continued to spread to different regions and across the seasons. In the present study, based on field and artificial assays, resistance to powdery mildew was identified in 6 annual and 13 perennial species. Annual species are preferred over perennials due to high crossability success and hybrid fertility. This investigation shows high resistance in *H. pauciflorus* (PAU-S-869) for the first time. The accessions DEC-1922, RIG-1696 and SAL-241 were found to be free of powdery mildew both in this study and that of Dedic et al. (2012), but the accessions DEC-1887, DIV-1881, DIV-1885, GIG-2017, GRA-2043, HIR-1536, MAX-33001 and TUB-15 reported as resistant in Dedic et al. (2012) were found to be susceptible in this study. Results of Dedic et al. (2012) were based on natural infection in the field, while in the present study, reaction of wild sunflowers to powdery mildew is based on both natural and artificial infection conditions. Even under natural infection conditions, some of the accessions (DEC-1887, DIV-1881, DIV-1885 and HIR-1536) showed contrasting reaction, which probably could be due to differences in the virulence of the pathogen or the conditions favouring natural infection in the two regions. In the present study, intra-accessional variability in the reaction to powdery mildew was found in *H. decapetalus*, *H. giganteus*, *H. grosseserratus*, *H. maximiliani*, *H. nuttallii*, *H. strumosus* and *H. tuberosus* and similar observations were reported by Christov (2008), Mc Carter (1993) and Dedic et al. (2012). Based on a varied reaction of *H. grosseserratus* and *H. maximiliani* collected from different locations to *Erysiphe cichoracearum*, Saliman et al. (1982) suggested that the

establishment of different segregated genotypes of these wild species in localized areas might lead to new genetic bases for varied responses to *E. cichoracearum*.

Based on visual rating, the disease score in wild species varied from 0 to 9. In few cases, there was no positive correlation between spore count and disease index. For example the species with disease index of 7 had a spore count that ranged from 22,000 to 37,000. Wild *Helianthus* species exhibit wide variability in vegetative and floral characters including leaf characters. Since powdery mildew penetration into the leaf mainly depends on leaf micro morphological characters, differential reaction of the species could be due to variations in the vegetative characters. Chattopadhyay et al. (2011) found highly significant and strong correlation between prevalence of powdery mildew and leaf characters *viz.,* stomatal index, stomatal density and trichome density, which were found causal for successful spore penetration of the leaf. As indicated by Fondevilla et al. (2007), diverse resistance offered by *Pisum* species was found to be governed by distinct cellular mechanisms; which probably could be due to several cellular mechanisms involved in plant-pathogen defense mechanisms. Likewise, characterization of defense responses in *Helianthus* species conferring resistance to *G. orontii* is necessary for understanding the mechanism of resistance and broadening the genetic base of cultivated sunflower for powdery mildew resistance.

Among the cultivar germplasm, 11% were moderately resistant to powdery mildew and none of the accessions studied were found to be immune. Four accessions (TX 16R, EC-537925, HIR-1734-2, ID-25) were found to be resistant to *G. orontii.* The accession PI 642072 (Reg no.GP-305) was also registered for its resistance to sunflower rust caused by *Puccinia helianthi* Schw, downy mildew caused by *Plasmopara halstedii* and sunflower mosaic virus (Jan and Gulya 2006). The interspecific derivative RES-834-3 (GP-155) is a derivative of *H. resinosus*. The present study for the first time shows high resistance in *H. resinosus* and it has been reported for its potential tolerance to stem rot. The interspecific derivative HIR-1734-2 (GP-151) is derived from *H. hirsutus* and is reported for its resistance to *Alternaria helianthi.* Being sources of multiple resistance, these accessions serve as potential donors in resistance breeding programmes in sunflower.

The range of AUPDC values among the germplasm lines ranged from 95 to 648, which indicate the importance of estimating the progress of disease in the germplasm. The positive correlation between DSI and AUDPC indicates the positive association between the two variables. The negative correlation between DSI and AUDPC with 50% days flowering

indicates the importance and association of flowering time with progress of the disease (except a weak positive correlation value of r = 0.07 between DSI and 50% flowering of core germplasm). The core germplasm provided a valuable resource for screening and understanding the progression of the disease and its association with leaf area and flowering duration. Since powdery mildew is a foliar disease that occurs at post flowering stages, it is important to consider disease progression with these parameters. The correlation values of leaf area with that of DSI and AUDPC varied from core germplasm (positive) and interspecific derivatives (negative), which indicates that the association is dependent on the genetic base and the extent of variability in the germplasm.

The present study identified new sources of resistance to sunflower powdery mildew in cultivar background, which could be utilized in breeding programmes aimed at resistance to powdery mildew. The study resulted in identification of a highly susceptible accession like PS 2023 derived from interspecific cross involving *H. petiolaris* that could be used as recipient parent in gene tagging and mapping studies as it aids in clear manifestation of the disease. Saliman et al. (1982) staggered the sowing of the susceptible hybrid sunflower cultivar 894 at 2-week intervals so that ten 2-week-old plants could be inoculated throughout the course of the study. The present study showed the high susceptibility of the perennial wild *Helianthus* species, *H. strumosus* (Acc. STR-15), which could be used as a continuous source of inoculum since powdery mildew fungus being are obligate ascomycete is difficult to be cultured and maintained under artificial conditions.

For powdery mildew, resistance cannot be relied on a single source as it has been proved that the resistance breaks down rapidly because of the coexistence of sexual and asexual reproduction cycles. Hence, genes responsible for partial resistance are potentially useful for development of cultivars with durable resistance. Other sources including wild species identified in this study are being investigated for their utility in the introgression of resistance to powdery mildew into agronomically desirable backgrounds. Yield losses due to powdery mildew are reported in several crops but have not been assessed systematically for sunflower. Since the disease is spreading in the warmer climates besides making its appearance during all growing seasons and all stages of the crop including cotyledonary leaves, it is important to make yield loss assessments.

Chapter 5

Histopathological study of the *G. orontii* infection process in sunflower and host defense responses to infection

The process of *G. orontii* infection in sunflower was studied. The histochemical parameters that occur during *G. orontii* infection process in sunflower was investigated. The changes in the levels of oxidative stress and antioxidant enzymes induced in both susceptible and resistant sunflower genotypes against *G. orontii* were measured for determining the involvement of their role in conferring host defense in response to powdery mildew infection and results and discussion were presented in the following chapter.

5.1. Results

5.1.1. Morphological features of *G. orontii* by light microscopy and scanning electron microscopy

To study the morphological details of *G. orontii*, whole leaf/stem samples of susceptible (PS 2023) genotypes were prepared and observed under light and scanning electron microscopy. Conidia were ovoid (dolliform) (Fig. 5.1 a and Fig. 5.2 a-i). Conidiophores were mostly erected with long foot cells followed by 3-5 short cells (conidia) (Fig. 5.1 b-d and Fig. 5.4 a, c). The size of the conidia was about 21-26 X 10-13 µm (Fig. 5.2 a, d, e, f). The conidial germ tube was moderately long and the germinating site is mainly terminal (Fig. 5.3 f-j). The length of germ tube was 1-2 times the width of the conidium and took 6-10 hours post infection (hpi) for full development (Fig. 5.3 f-j). The conidia showed a dense; honey combed wrinkling on collapsed conidia (Fig. 5.4 b). Conidia were without fibrosin bodies (Reticuloidium type). The germinal tubes ended in a club-shaped or slightly swollen appressorium and nipple appressoria on superficial hyphae (Fig. 5.4 a-c).

5.1.2. Penetration of *G. orontii* in sunflower

Conidial germination occurred within six hours of inoculation (Fig. 5.1 a), increased at 12 hours and reached maximum at 48 hours. The penetration of germ tubes occurred mostly through stomata (Fig. 5.3 e-g) and hyphae were found growing towards the stomata (Fig. 5.3 i-k). The penetration of germinated conidia also occurred through the junctions of epidermal cells (Fig. 5.3 a, b) and penetration through mechanical damage was rarely observed (Fig. 5.3 c, d). The formation of a unilobed appressorium was observed at the penetration sites (Fig. 5.4 a) and superficial nipple shaped appressoria were observed on the hyphae (Fig. 5.4 b, c). Host cell penetration with internal mycelium was observed in epidermal cells (Fig. 5.4 e, f). After successful penetration and at 5-6 days post infection (dpi), young conidiophores emerged from stomata (Fig. 5.3 i). Infection and development of conidiophores of *G. orontii* were also observed on stems of sunflower (Fig. 5.4 h, i).

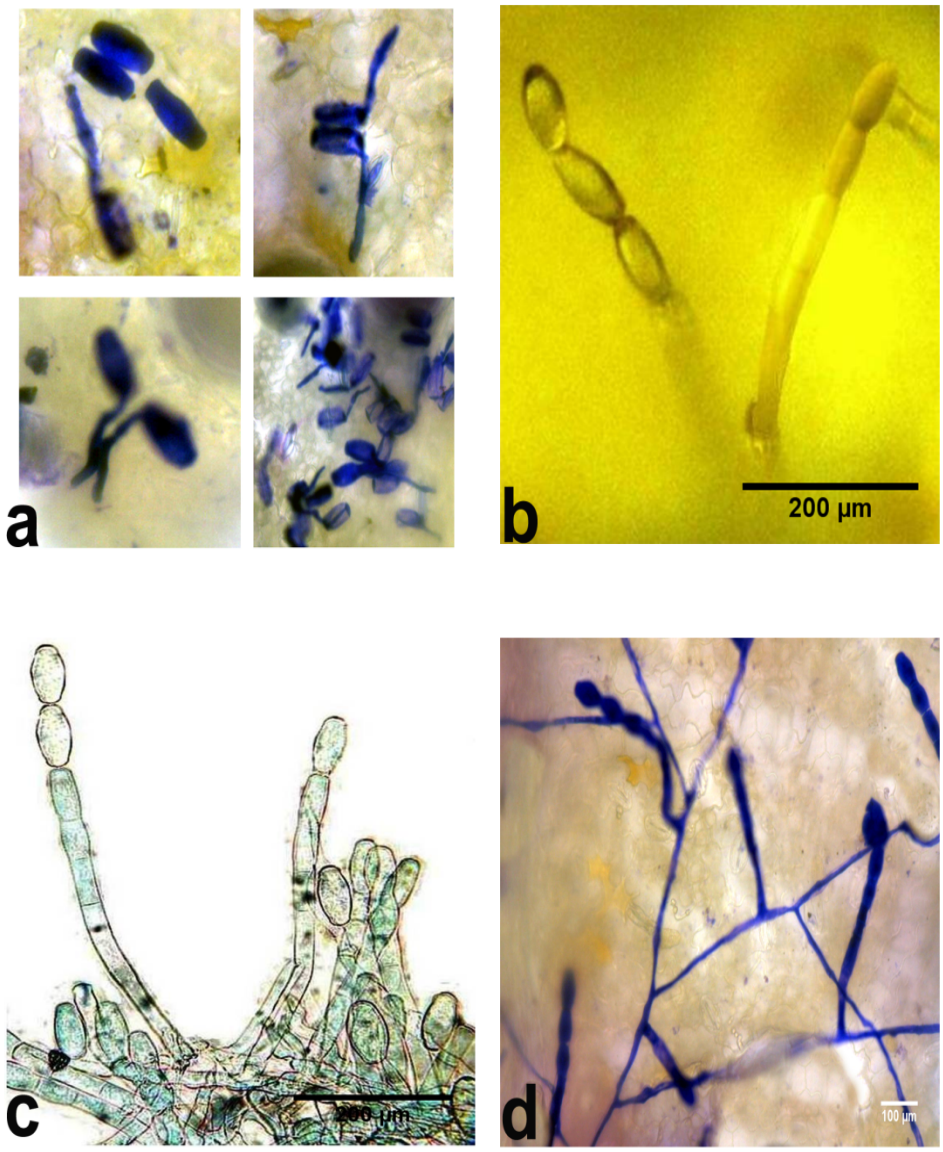

Fig. 5.1 Morphological features of *G. orontii* on infected leaves of PS 2023 (susceptible genotype) by light microscopy (Figure 5.1 a dolliform conidia along with terminal germ tube and Figure 5.1 b-d erected conidiophores with 3-5 short conidia).

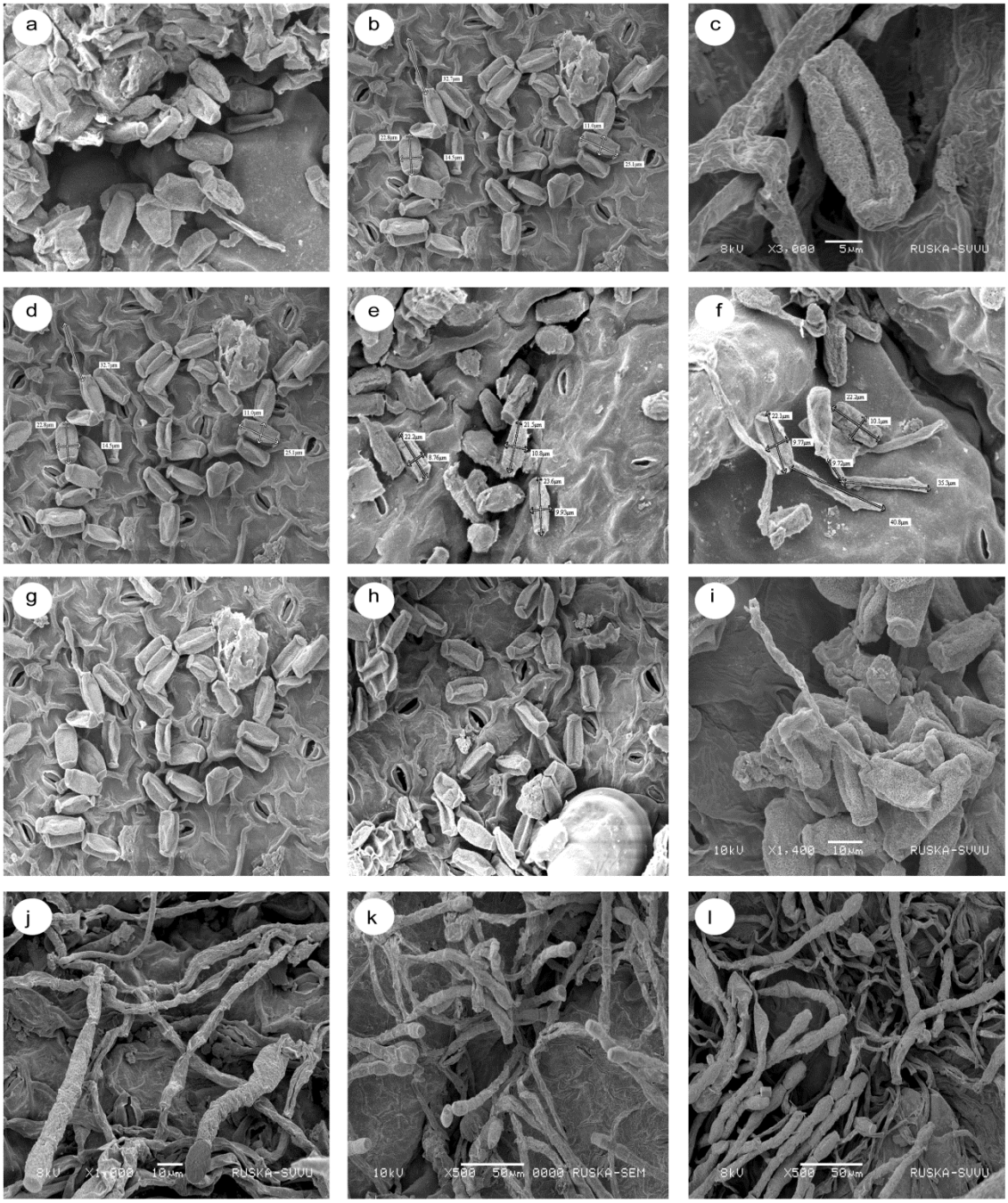

Fig. 5.2 Representative morphological features of *G. orontii* on infected leaves of PS 2023 (susceptible genotype) by SEM (5.2 a-i dolliform conidia along with terminal germ tube, 5.2 d-f measurement of conidial dimensions and 5.2 j-i erected conidiophores with 3-5 short conidia).

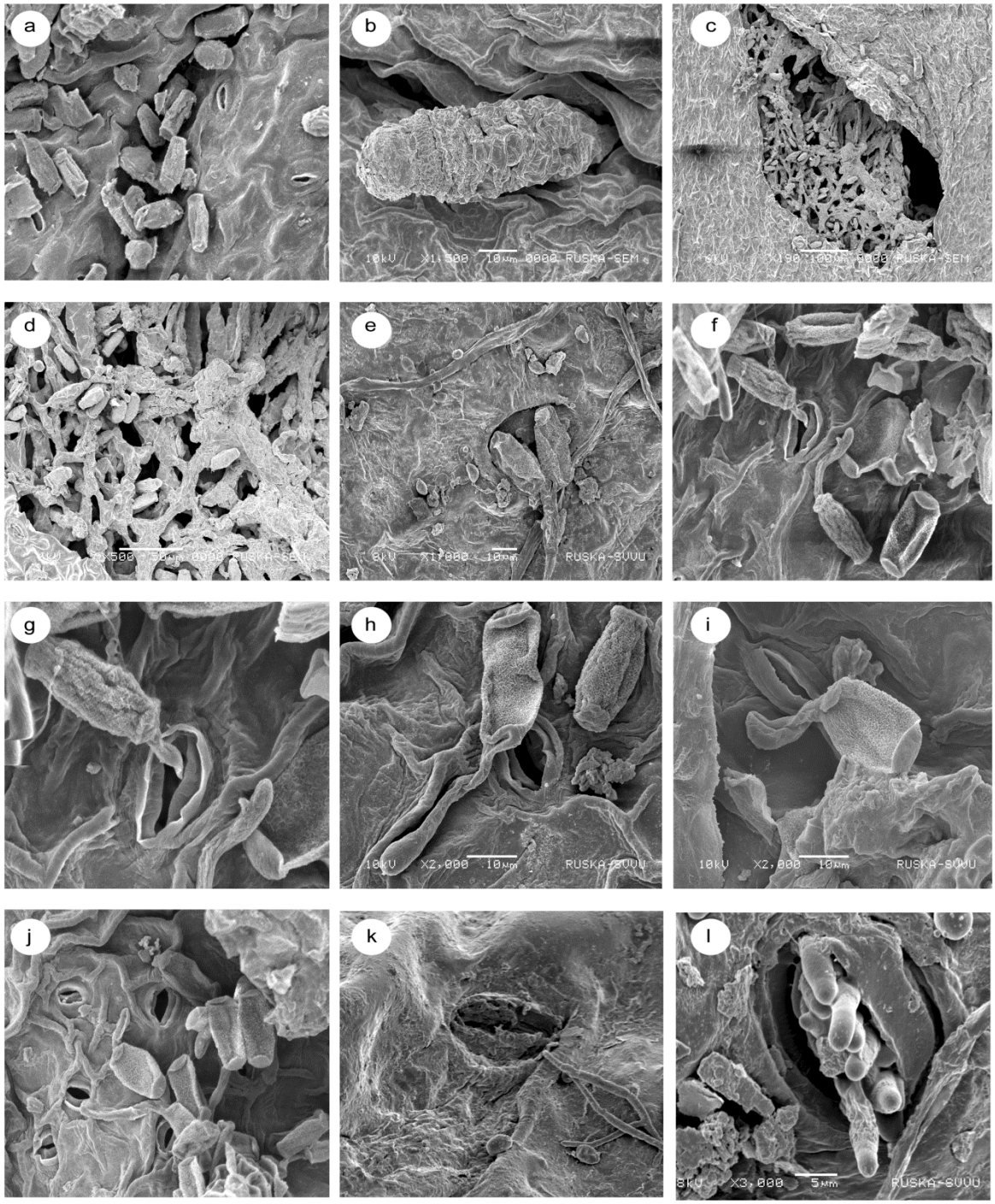

Fig. 5.3 Scanning electron micrographs of *G. orontii* conidial germination, infection structure formation, and mode penetration on leaf of PS 2023 (5.3 a-b penetration of *G. orontii* into sunflower through junction between epidermal cells, 5.3 c-d penetration of *G. orontii* into sunflower through the damage created by mechanical injury, 5.3 e-k penetration of *G. orontii* into sunflower through stomata openings, and 5.3 i emergence of conidiophores from stomata openings).

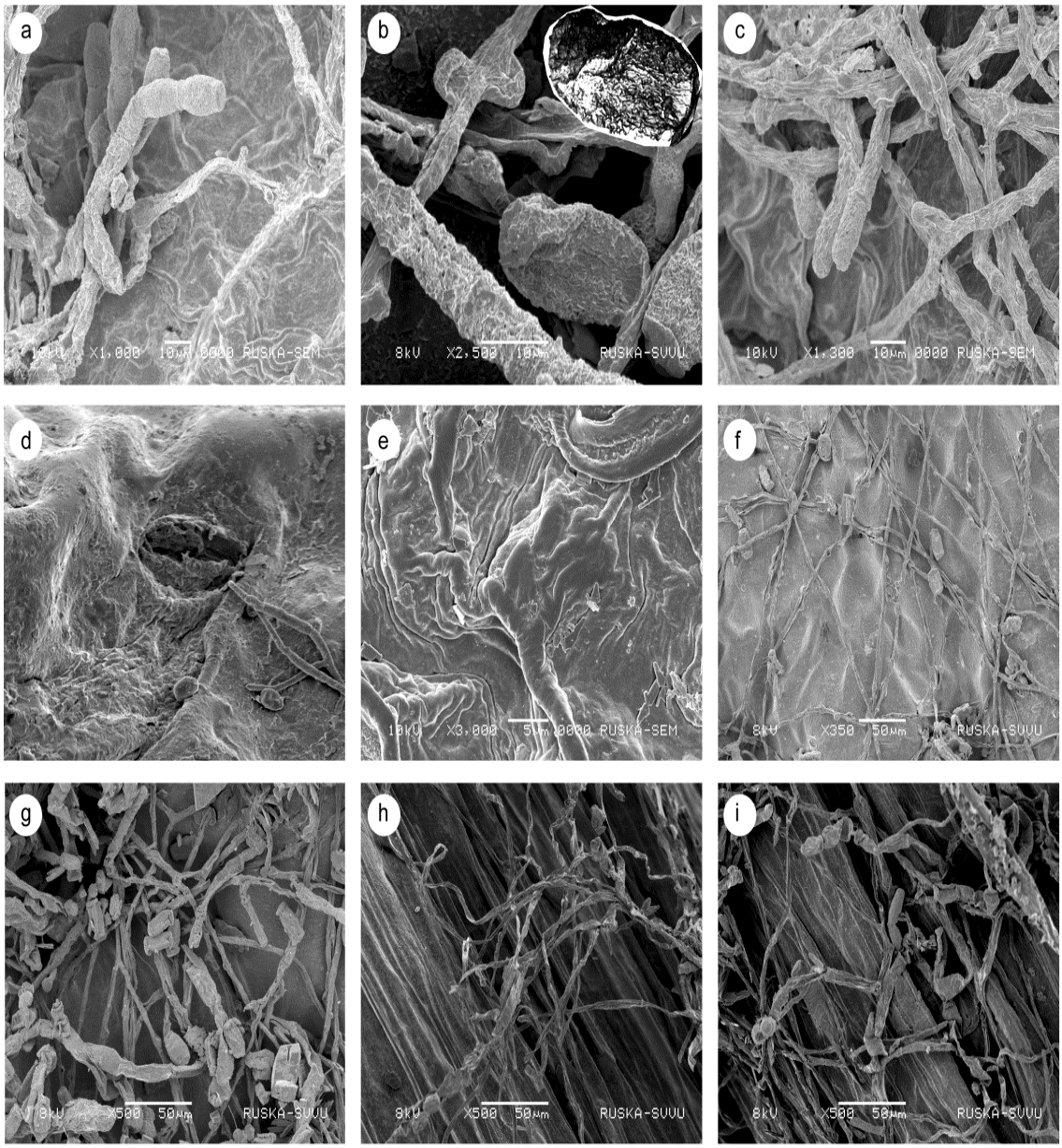

Fig. 5.4 Scanning electron micrographs of *G. orontii* on infected leaves of PS 2023 representing the appressorium formation at the end of germ tube (5.4 a), superficial nipple shaped appressorium (5.4 b, c), Hyphae penetration and internal mycelium (5.4 d-g) and *G. orontii* on stem (5.4 h-i).

5.1.3. Light microscopic analysis of the development of powdery mildew

Microscopic analysis of the development of powdery mildew showed infection within six hours of inoculation with enhanced hyphal growth and penetration in susceptible genotypes when compared with the resistant genotypes in which hyphal growth was sparse and observed only after 3 dpi (Fig. 5.5 and 5.6). The differences between genotypes were discernible at 1dpi and increased until 10 dpi. In susceptible genotypes (PS 2023 and Morden), the hyphal growth increased rapidly and with a maximum spread at 7 dpi (Fig. 5.5), where as TX 16R and ID-25 (resistant) genotypes showed resistance to *G. orontii* penetration and allowed less hyphal expansion (Fig. 5.6). Microscopic observations showed that the resistance exhibited by the two resistant (TX 16R and ID-25) genotypes was characterized by moderate rates of entry into host epidermal cell resulting in reduced formation of conidiophores (Fig. 5.6). *G. orontii* developed extensive hyphal growth and covered the entire leaf surface on both susceptible genotypes (PS 2023 and Morden) (Fig. 5.5).

5.1.4. Conidiophores count

An estimate of the number of conidiophores was performed at 7 dpi in all the four genotypes. Abundant conidiophores were observed in susceptible genotypes (Fig. 5.5); however, the hyphal structures and conidiophores produced on resistant genotypes (TX16R and ID-25) were reduced in number (Fig. 5.6). The resistance to *G. orontii* in resistant genotypes (TX 16R and ID-25) was observed at a later stage in the infection process (7 dpi). The numbers of conidiophores was decreased. The drastic reduction in number of conidiophores at 7 dpi in resistant genotypes (Fig. 5.7) might be because of the onset of resistance against powdery mildew (4-5 dpi).

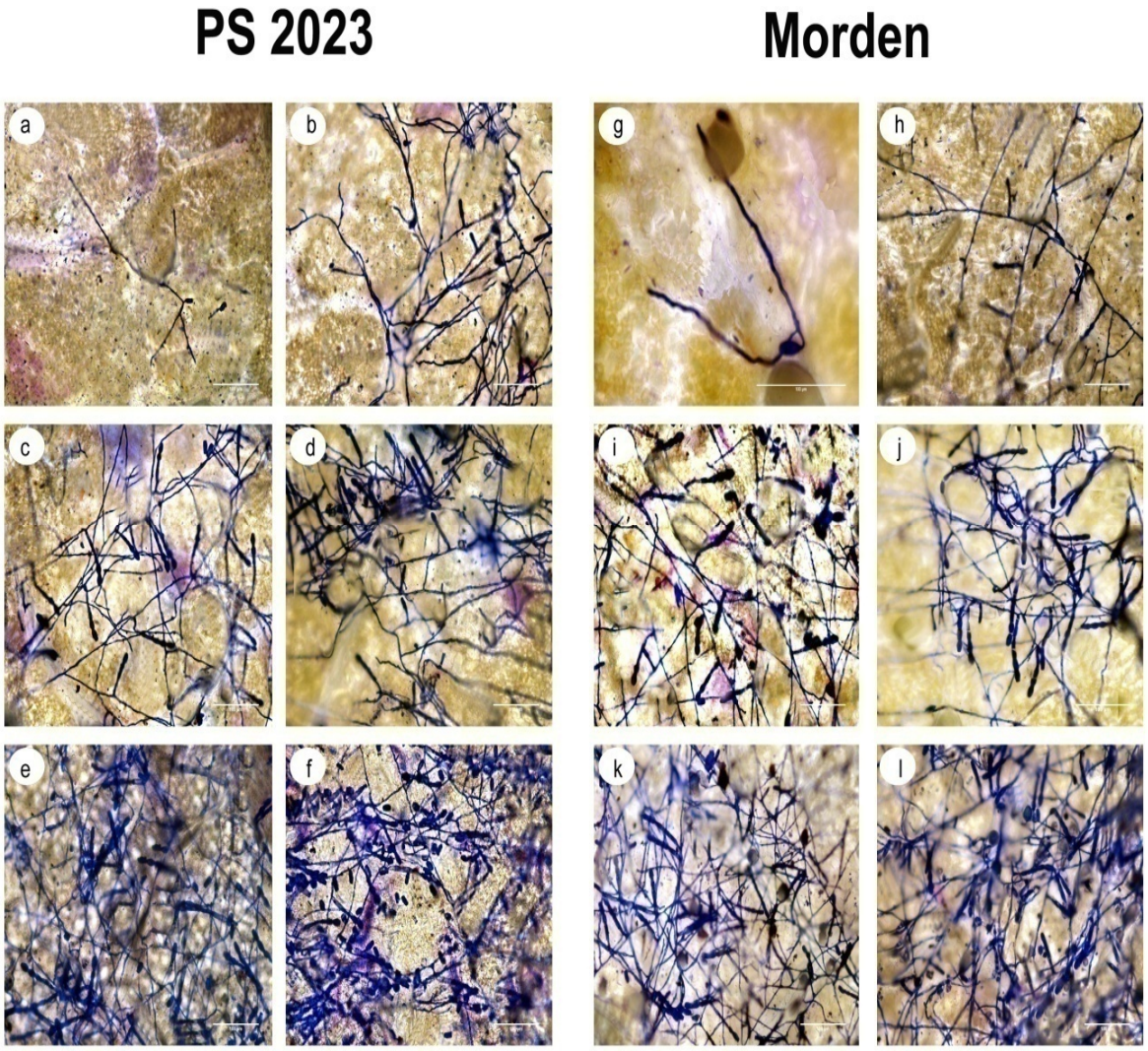

Fig. 5.5 The light microscopy micrographs show the expansion of the *G. orontii* colony on the surface of susceptible genotypes i.e PS 2023, Morden at 1-10 dpi (a-1 dpi, b-3 dpi, c-5 dpi, d-7 dpi, e-9 dpi and f-10 dpi).

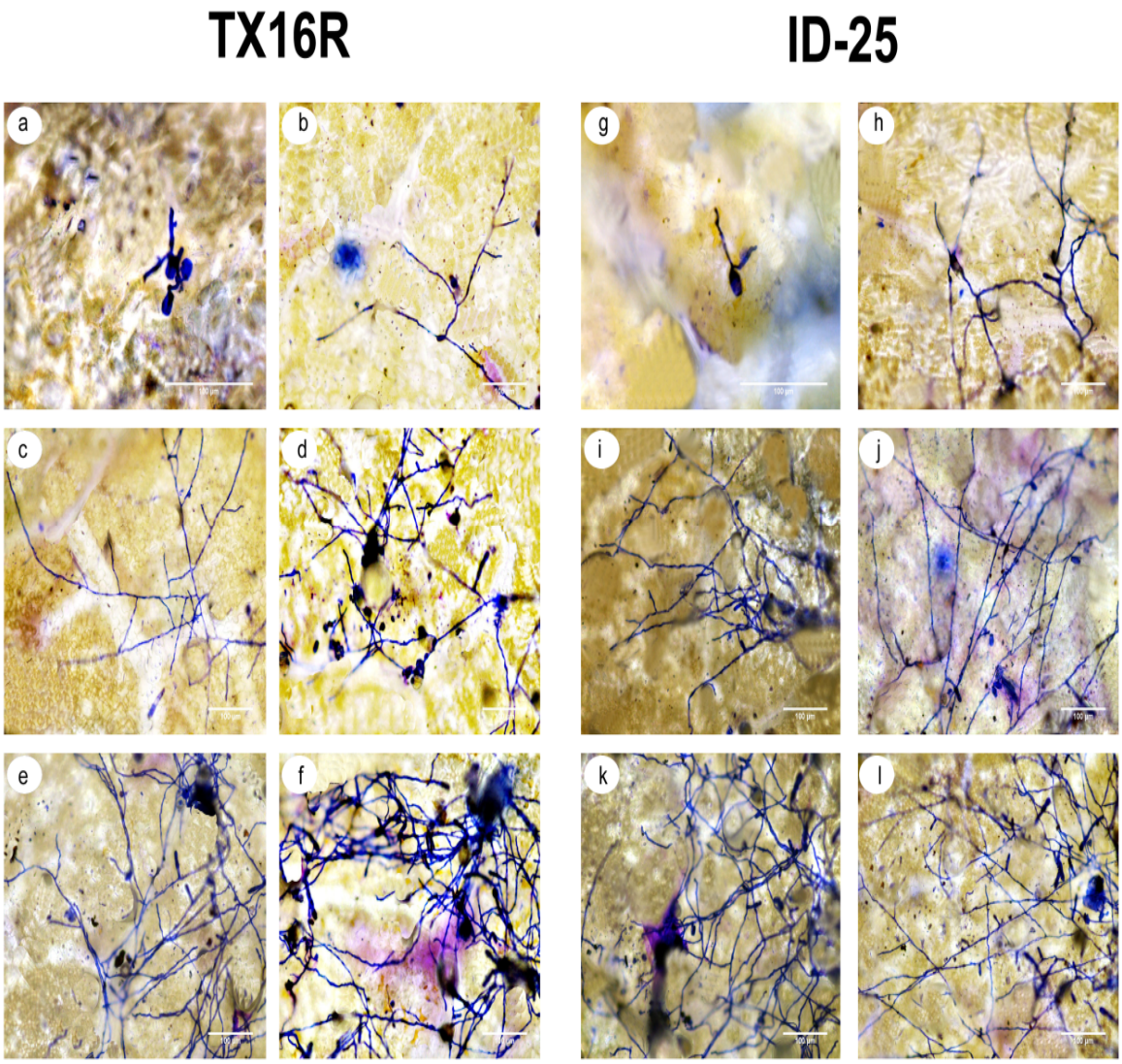

Fig. 5.6. The light microscopy micrographs show the expansion of the *G. orontii* colony on the surface of resistant genotypes i.e TX 16R, ID-25 at 1-10 dpi (a-1 dpi, b-3 dpi, c-5 dpi, d-7 dpi, e-9 dpi and f-10 dpi).

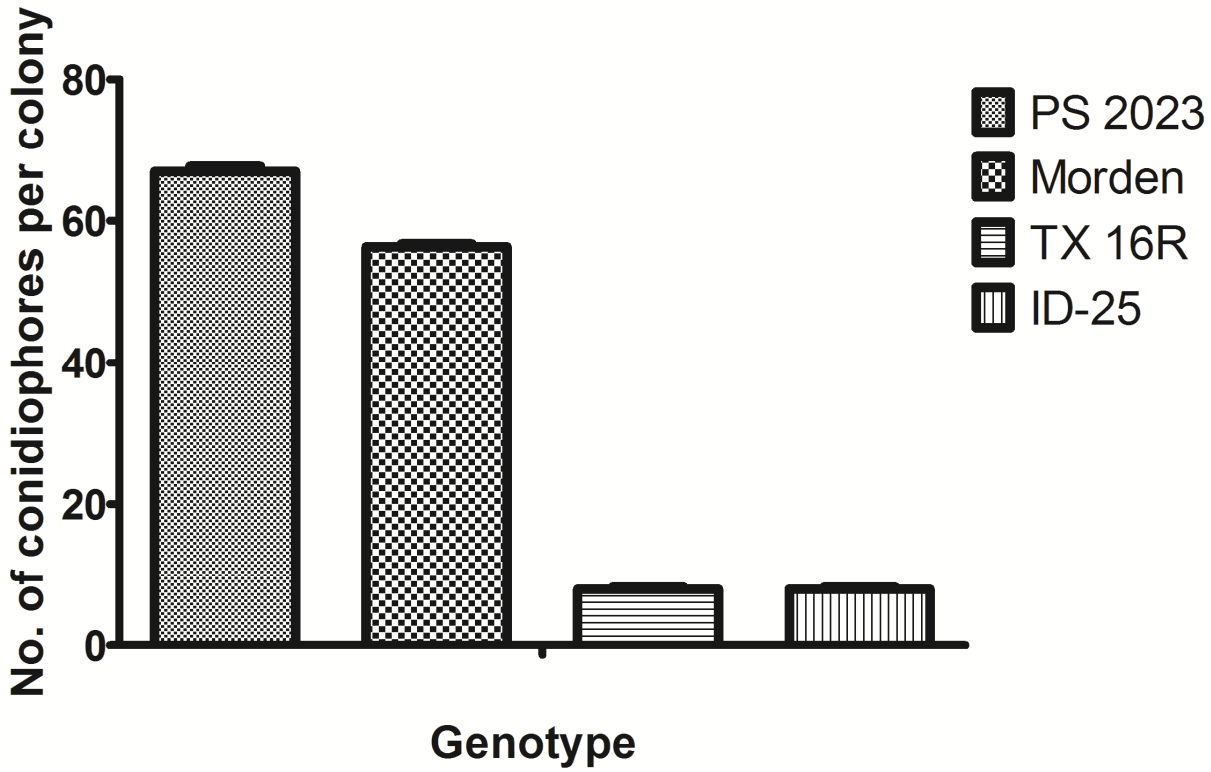

Fig. 5.7. Count of powdery mildew conidiophores on susceptible (PS 2023, Morden) and resistant (TX 16R, ID-25) genotypes at 7 dpi.

5.1.5. Histochemical localization of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2)

Temporal evolution of H_2O_2 and O_2^- and their accumulation in susceptible and resistant genotypes was studied at different time points post inoculation (1-10 dpi). Localization of H_2O_2 was observed in the sunflower leaves as a red-brown precipitate after DAB staining (Fig. 5.8, 5.9, 5.10, 5.11). A weak staining was observed in control (uninoculated) leaves. In susceptible genotypes (PS 2023 and Morden) infected with powdery mildew, the staining was much more intense compared to the control and was spread all over the leaf (Fig. 5.8 and 5.9). In the resistant genotypes, H_2O_2 was observed with low spread of the staining over the leaves. In resistant genotypes, H_2O_2 was observed with low spread of the staining over the leaves (Fig. 5.10 and Fig. 5.11).

In both susceptible and resistant genotypes, there was accumulation of H_2O_2. However, a very weak DAB staining was observed in the leaves of susceptible genotypes at initial infection stages. Later on, the spread of powdery mildew occurred vigorously upon in the intercellular and cells in contact with intercellular hyphae prolonged incubation forming houstoria with intercellular hyphae and showed DAB staining. Quantitative levels of H_2O_2 were also determined spectrophotometrically as well. Macroscopically visible blue colour indicates the accumulation of O_2^-. The cellular localization of O_2^- was detected by NBT staining. The reduction of NBT to dark blue formazan by superoxide radicals results in blue coloration on leaves**.** The trends of O_2^- accumulation in susceptible and resistant genotypes followed the same pattern as that of H_2O_2 (Fig. 5.8, 5.9, 5.10, 5.11).

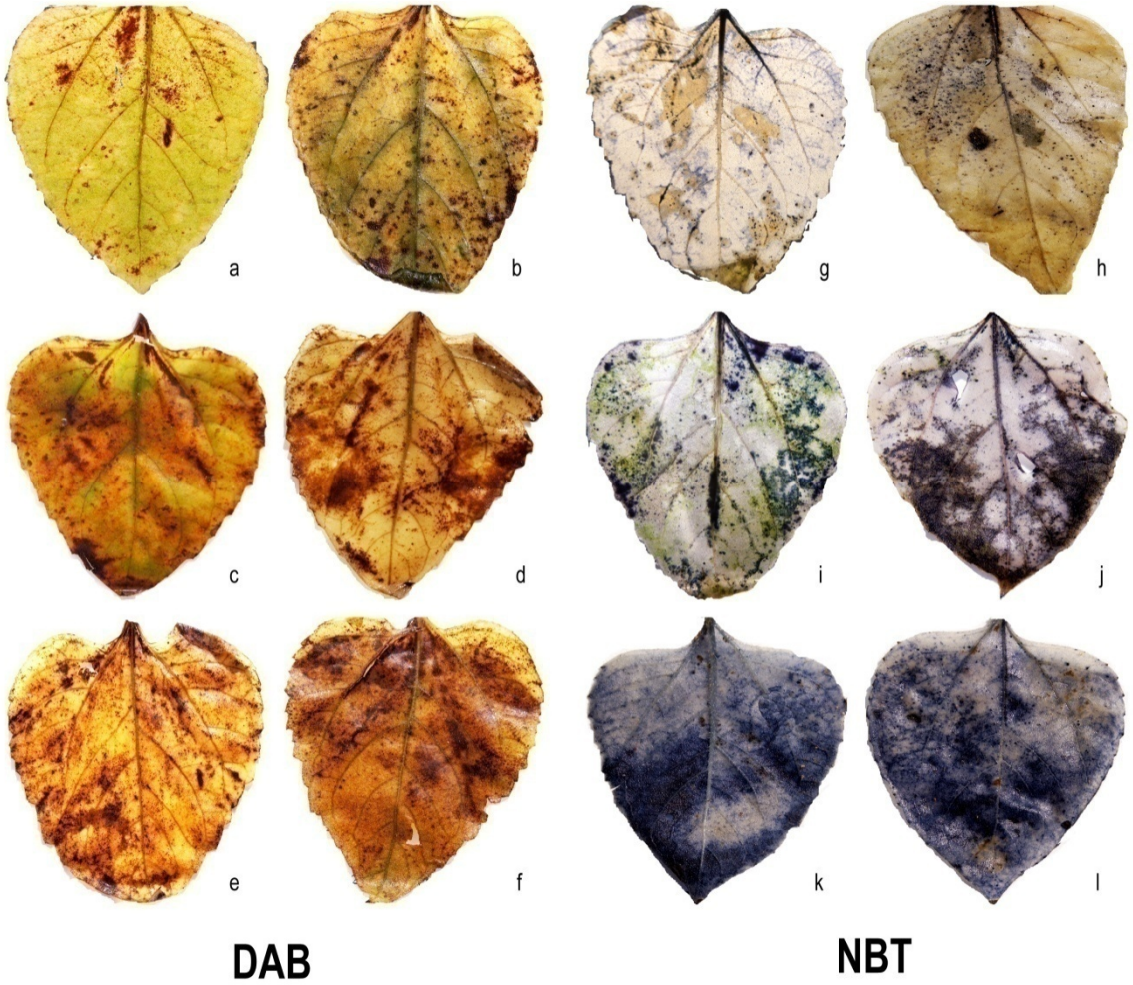

Fig. 5.8 Accumulation of H_2O_2 (brown colour) and O_2^- (blue colour) within leaves of powdery mildew infected genotypes PS 2023 during 1-10 dpi (a-1 dpi, b-3 dpi, c-5 dpi, d-7 dpi, e-9 dpi and f-10 dpi).

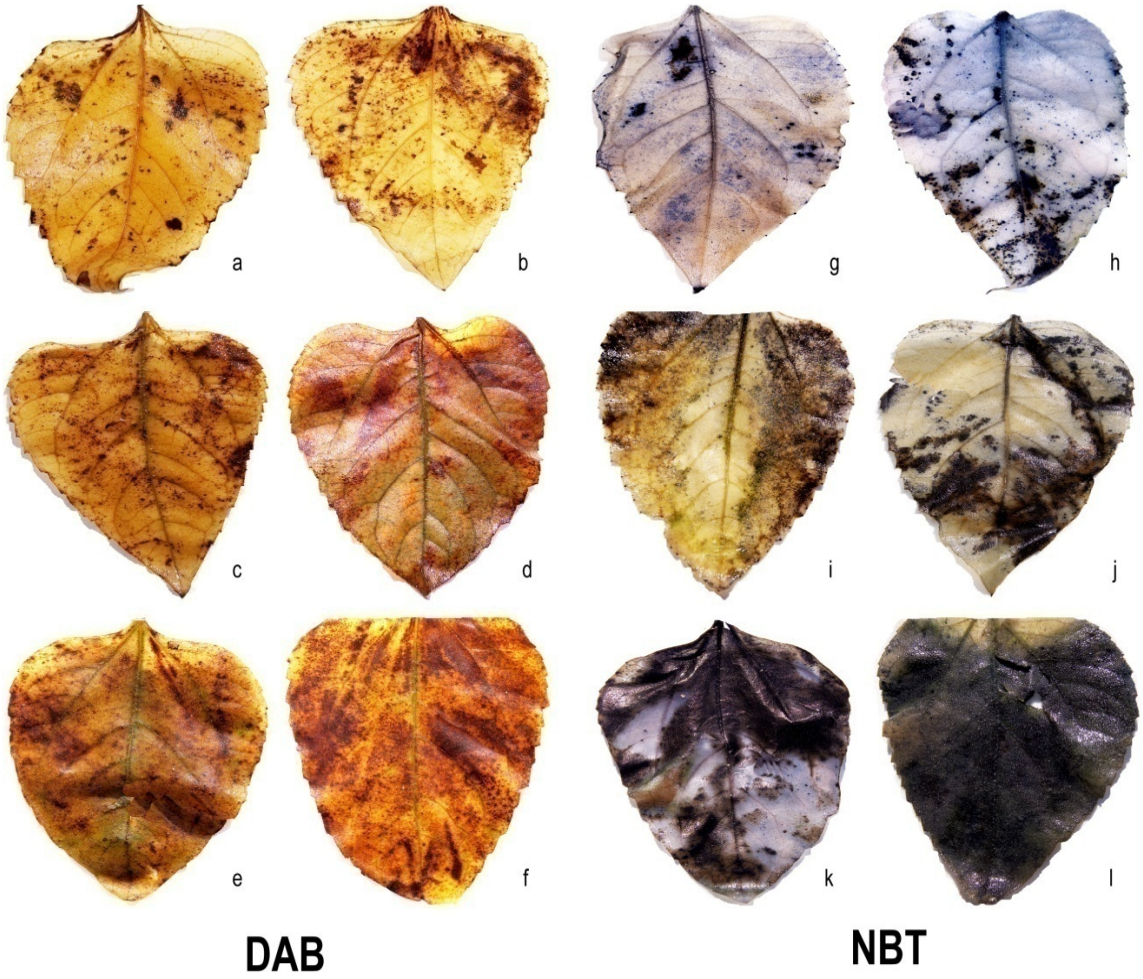

Fig. 5.9 Accumulation of H_2O_2 (brown colour) and O_2^- (blue colour) within leaves of powdery mildew infected genotypes Morden during 1-10 dpi (a-1 dpi, b-3 dpi, c-5 dpi, d-7 dpi, e-9 dpi and f-10 dpi).

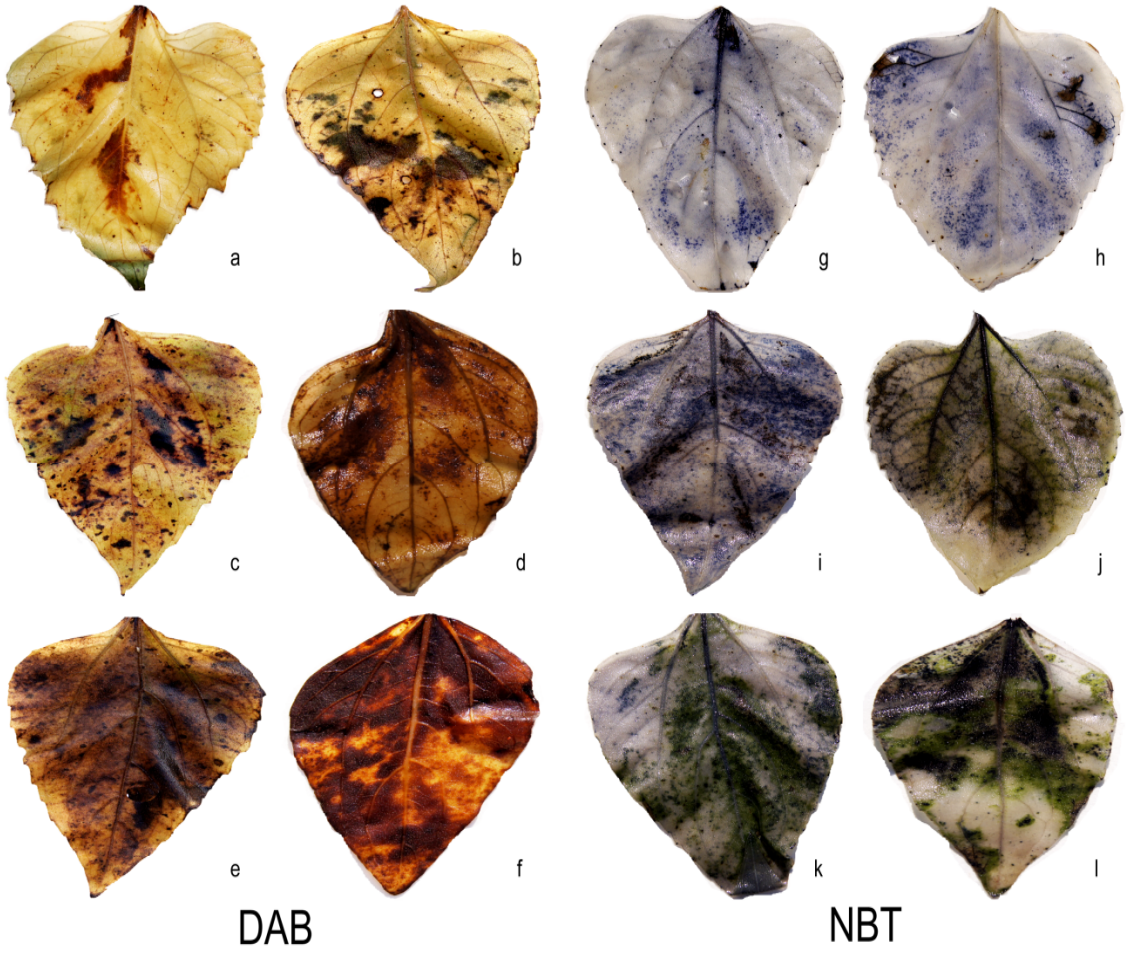

Fig. 5.10 Accumulation of H_2O_2 (brown colour) and O_2^- (blue colour) within leaves of powdery mildew infected genotypes ID-25 during 1-10 dpi (a-1 dpi, b-3 dpi, c-5 dpi, d-7 dpi, e-9 dpi and f-10 dpi).

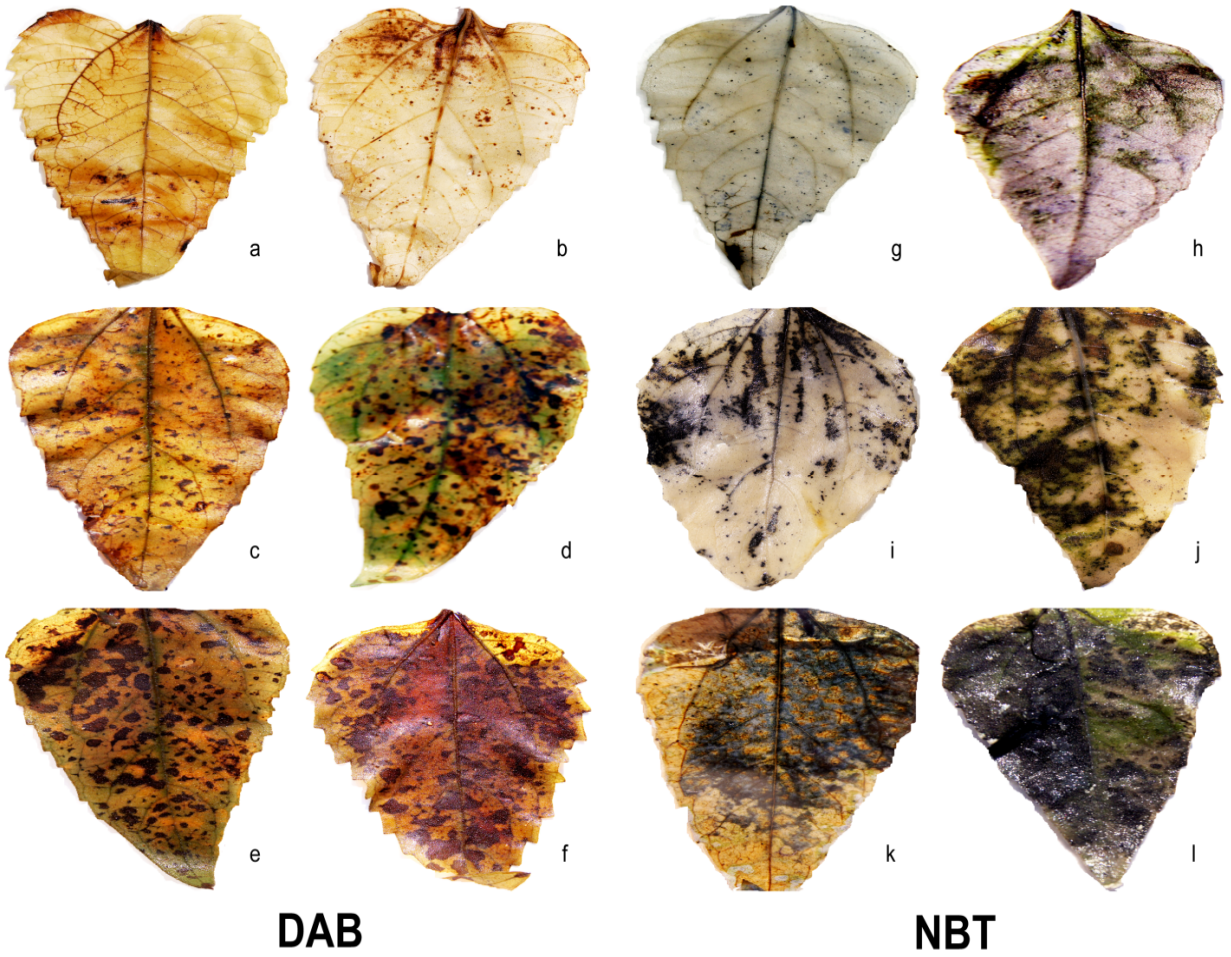

Fig. 5.11 Accumulation of H_2O_2 (brown colour) and O_2^- (blue colour) within leaves of powdery mildew infected genotypes TX 16R during 1-10 dpi (a-1 dpi, b-3 dpi, c-5 dpi, d-7 dpi, e-9 dpi and f-10 dpi).

5.1.6. Quantification hydrogen peroxide (H_2O_2)

The Figure 5.12 represents the results on the accumulation of H_2O_2, a reactive oxygen species that is toxic for cell macromolecules. The data revealed a progressive increase of H_2O_2 content from 1dpi to 10 dpi in all the genotypes. The levels of H_2O_2 in powdery mildew inoculated Morden range from 1851.30 ± 12.44 μmol g^{-1} l wt. (1 dpi) to 6693.09 ± 46.85 μmol g^{-1} l wt. (10 dpi) and in PS 2023, it ranges from 2075.99 ± 12.44 μmol g^{-1} l wt. (1 dpi) to 6361.43 ± 44.05 μmol g^{-1} l wt. (10 dpi). In resistant genotypes, the levels of H_2O_2 in powdery mildew inoculated TX 16R ranged from 1823.34 ± 12.24 μmol g^{-1} l wt. (1 dpi) to 5526.96 ± 40.07 μmol g^{-1} l wt. (10 dpi) and from 1354.35 ± 8.91 μmol g^{-1} l wt. (1 dpi) to 4333.41 ± 30.79 μmol g^{-1} l wt. (10 dpi) in ID-25 (Table 5.1). During the infection process, H_2O_2 produced was much higher in susceptible genotypes than the resistant ones. The enzyme ascorbate peroxidase (APX) also detoxifies H_2O_2 and the level of APX activity was manifold higher in the resistant cultivars, which may have resulted in faster depletion of H_2O_2, which has resulted in their resistance phenomenon.

5.1.7. Antioxidant enzymes

5.1.7.1. Superoxide dismutase (SOD)

The activities of superoxide dismutase (SOD) in leaves of genotypes susceptible and tolerant to powdery mildew that were subjected to pathogen infection are presented in Figure 5.13. The levels of SOD in powdery mildew inoculated Morden ranged from 418.65 nmol g^{-1} l wt. (10 dpi) to 921.77 ± 6.55 nmol g^{-1} l wt. (4 dpi) and in PS 2023, it ranged from 408.30 ± 4.32 nmol g^{-1} l wt. (10 dpi) to 827.38 ± 5.88 nmol g^{-1} l wt. (5 dpi). In resistant genotypes, the levels of H_2O_2 in powdery mildew inoculated TX 16R ranged from 562.55 ± 3.99 nmol g^{-1} l wt. (9 dpi) to 1086.83 ± 9.14 nmol g^{-1} l wt. (4 dpi) and in ID-25, it ranged from 601.05 ± 4.27 nmol g^{-1} l wt. (10 dpi) to 1049.87 ± 8.87 nmol g^{-1} l wt. (10 dpi) (Table 5.2). The basal level of SOD activity in leaves was observed to be significantly higher in powdery mildew resistant genotypes compared to the powdery mildew susceptible genotypes. In control, SOD activity increased continuously throughout the infection period in all the genotypes. However, the increase was more pronounced in resistant genotypes as compared to the susceptible genotypes. In

contrast, in infected leaves the activity increased up to 4 dpi and thereafter decreased sharply in all the genotypes. However, the resistant genotypes exhibited higher SOD activity at all the stages of infection, thus dismutating O_2^- anion efficiently.

5.1.7.2. Peroxidase (POX)

The Figure 5.14 represents the differential effect of infection on peroxidase activity in powdery mildew susceptible and resistant sunflower genotypes. The levels of POX in powdery mildew inoculated Morden ranged from 90.99 ± 0.06 nmol g^{-1} l wt. (10 dpi) to 1095.47 ± 7.78 nmol g^{-1} l wt. (6 dpi) and in PS 2023, it ranged from 221.04 ± 0.85 nmol g^{-1} l wt. (10 dpi) to 1914.04 ± 13.60 nmol g^{-1} l wt. (6 dpi). In resistant genotypes, the levels of H_2O_2 in powdery mildew inoculated TX 16R ranges from 362.88 ± 1.86 nmol g^{-1} l wt. (1 dpi) to 1459.50 ± 10.37 nmol g^{-1} l wt. (5 dpi) and in ID-25, it ranged from 536.24 ± 5.23 nmol g^{-1} l wt. (10 dpi) to 1854.82 ± 13.18 nmol g^{-1} l wt. (10 dpi). Regardless of the genotype, POX in the control plants activity was minimum at 1 dpi stage in all the genotypes and increased continuously during infection attaining the maximum value at the 10 dpi (Table 5.3). However, in infected plants of all the genotypes, highest enzyme activity recorded at 6 dpi, which decreased gradually at 10 dpi (Fig. 11 c).

5.1.7.3. Ascorbate peroxidase (APX)

The response of APX activity in leaves of powdery mildew susceptible and tolerant sunflower genotypes was presented in Figure 5.15. The levels of APX in powdery mildew inoculated morden ranges from 490.97 ± 4.91 nmol g^{-1} l wt. (1 dpi) to 1943.59 ± 13.10 nmol g^{-1} l wt. (10 dpi) and in PS 2023, it ranges from 302.15 ± 1.43 nmol g^{-1} l wt. (1 dpi) to 2141.61 ± 15.21 nmol g^{-1} l wt. (10 dpi). In resistant genotypes, the levels of APX in powdery mildew inoculated TX 16R ranged from 592.26 ± 4.20 nmol g^{-1} l wt. (1 dpi) to 3199.58 ± 24.15 nmol g^{-1} l wt. (10 dpi) and in ID-25, it ranged from 488.52 ± 2.76 nmol g^{-1} l wt. (1 dpi) to 2582 ± 19.77 nmol g^{-1} l wt. (10 dpi) (Table 5.4). APX activity increased linearly in tolerant and susceptible genotypes with severity of infection and also the days post infection. However, the magnitude of increase was more pronounced in the tolerant than in susceptible genotypes. In all the genotypes, infected leaves displayed very high APX activity as compared to their respective controls.

5.1.7.4. Glutathione reductase (GR)

Figure 5.16 represent the effect of the pathogen on GR activity in the leaves of powdery mildew susceptible and powdery mildew tolerant sunflower genotypes. The levels of APX in powdery mildew inoculated morden ranged from 772.21 ± 1.40 nmol g^{-1} l wt. (10 dpi) to 2576.35 ± 1.40 nmol g^{-1} l wt. (1 dpi) and in PS 2023, it ranged from 527.34 ± 1.37 nmol g^{-1} l wt. (10 dpi) to 2621.90 ± 1.37 nmol g^{-1} l wt. (1 dpi). In resistant genotypes, the levels of APX in powdery mildew inoculated TX 16R ranges from 1441.71 ± 1.40 nmol g^{-1} l wt. (10 dpi) to 3090.47 ± 2.30 nmol g^{-1} l wt. (1 dpi) and in ID-25, it ranged from 1425.58 ± 1.40 nmol g^{-1} l wt. (10 dpi) to 2882.45 ± 2.30 nmol g^{-1} l wt. (1 dpi). The initial level of GR activity in leaves was observed to be significantly higher in resistant genotypes than in susceptible genotypes (Table 5.5). Significant and progressive decrease with increase in infection period was observed in GR activity in resistant and susceptible genotypes. However, the decrease was more evident in susceptible genotypes as compared to the resistant genotypes.

Table 5.1 Hydrogen peroxide (H_2O_2) Concentration (µmols/ gram leaf wt.)

Genotype	Condition	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
Morden	**Control**	1334.27±9.47	1468.03±10.43	1689.86±12	1743.58±12.39	1838.3±13.06	2012.83±14.3	2274.32±15.45	2403.3±18.49	2566.42±19.66	2758.9±21.02
	Infected	1851.3±12.44	2505.94±17.09	3458.53±23.86	4535.09±31.52	5427.32±37.86	5736.7±40.05	6059.67±42.35	6196.14±43.32	6435.92±45.02	6693.09±46.85
PS 2023	**Control**	1713.89±11.46	1879.07±13.35	1885.6±13.39	2174.07±15.45	2210.89±15.71	2409.2±17.12	2576.05±18.3	2682.23±20.48	2678.97±20.45	2879.6±21.88
	Infected	2075.99±16.17	2412.87±17.14	2919.75±20.75	2985±21.21	3312.31±23.53	3860.38±27.43	4300.39±30.56	4932±34.33	5625.24±39.26	6361.43±44.5
TX 16R	**Control**	1415.39±9.34	1613.89±11.46	1975.32±14.04	2012.88±14.3	2112.38±15.01	2175.07±15.45	2301.23±16.35	2534.26±19.43	2559.13±19.6	2618.83±20.03
	Infected	1823.34±12.24	2113.88±14.31	2468.46±17.53	2608.89±18.54	2917.34±20.73	3494.44±24.83	3812.84±27.09	4289.44±30.48	4907.33±36.29	5526.96±40.7
	Control	1080.53±6.96	1163.5±7.55	1315.33±10.76	1527.38±12.27	1574.69±12.61	1713.33±13.59	1823.58±12.95	2031.96±14.44	2147.1±15.25	2148.22±15.26
ID-25	**Infected**	1354.35±8.91	1586.65±12.69	2097.74±16.32	2320.12±17.9	2640.37±20.18	3049.7±21.67	3559.16±25.29	3855.48±27.39	4246.42±30.17	4333.41±30.79

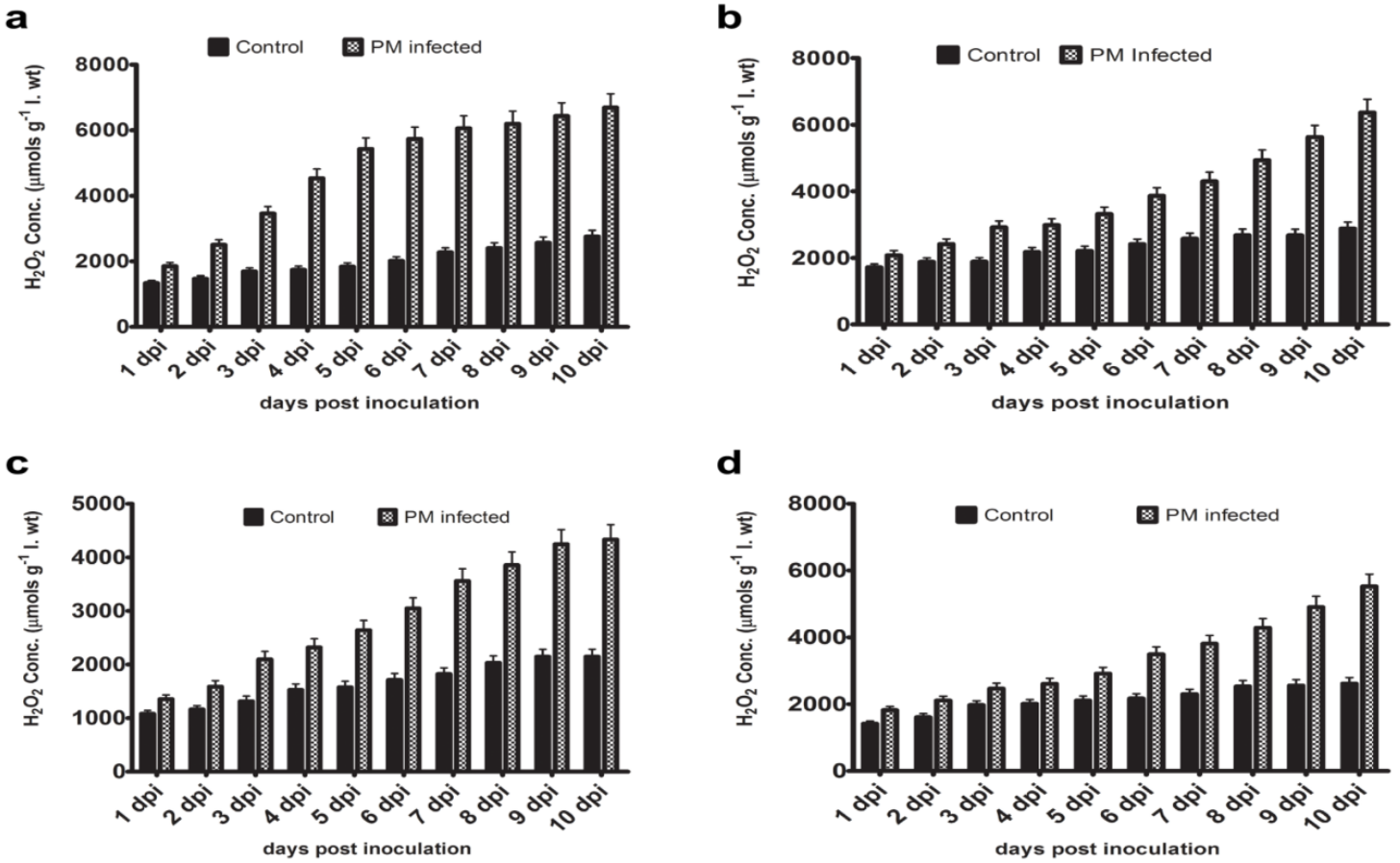

Fig. 5.12 Levels of H_2O_2 (µmols/ gram leaf. wt) in leaves of the susceptible (a-PS 2023 and b. Morden) and resistant (c-ID-25 and d- TX 16R)genotypes.

Table 5.2 Superoxide dismutase (SOD) Concentration (nmols /gram leaf. wt.)

Genotype	Condition	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
Morden	**Control**	601.32±3.56	617.51±4.39	633.5±4.5	650.71±4.62	667.41±4.74	681.19±4.84	699.94±4.97	720.07±5.11	737.21±6.65	764.76±6.85
	Infected	731.21±6.61	749.07±6.74	809.03±7.16	921.77±6.55	764.1±5.43	618.68±4.39	571.48±4.06	513.06±3.64	471.72±2.64	418.65±2.26
PS 2023	**Control**	469.66±2.62	485.82±3.45	502.2±3.56	533.43±3.79	553.58±3.93	575.16±4.08	588.55±5.6	610.41±5.75	629.37±5.89	650.47±6.04
	Infected	582.44±3.42	630.63±3.77	695.72±4.23	802.63±5.7	827.38±5.88	778.14±5.52	691.9±4.91	641.39±4.56	564.1±5.43	408.3±4.32
TX 16R	**Control**	781.44±4.84	829.63±5.18	854.42±6.07	868.3±6.17	882.9±6.27	910.55±6.47	938.47±6.66	959.27±8.23	977.54±8.36	1002.44±8.54
	Infected	826.89±7.29	922.56±7.97	962.1±8.25	1086.83±9.14	867.43±6.16	730.89±5.19	682.36±4.84	615.27±4.37	562.55±3.99	585.79±4.16
	Control	773.03±5.49	838.09±5.95	860.29±6.11	882.48±6.26	905.82±7.85	957.03±8.22	988.9±8.44	1023.34±6.56	1052.29±6.76	1084.74±6.99
ID-25	**Infected**	851.35±5.34	902.88±5.7	955.47±8.21	1049.87±8.87	974.86±8.34	896.77±6.37	816.4±5.79	772.01±5.48	742.42±5.27	601.05±4.27

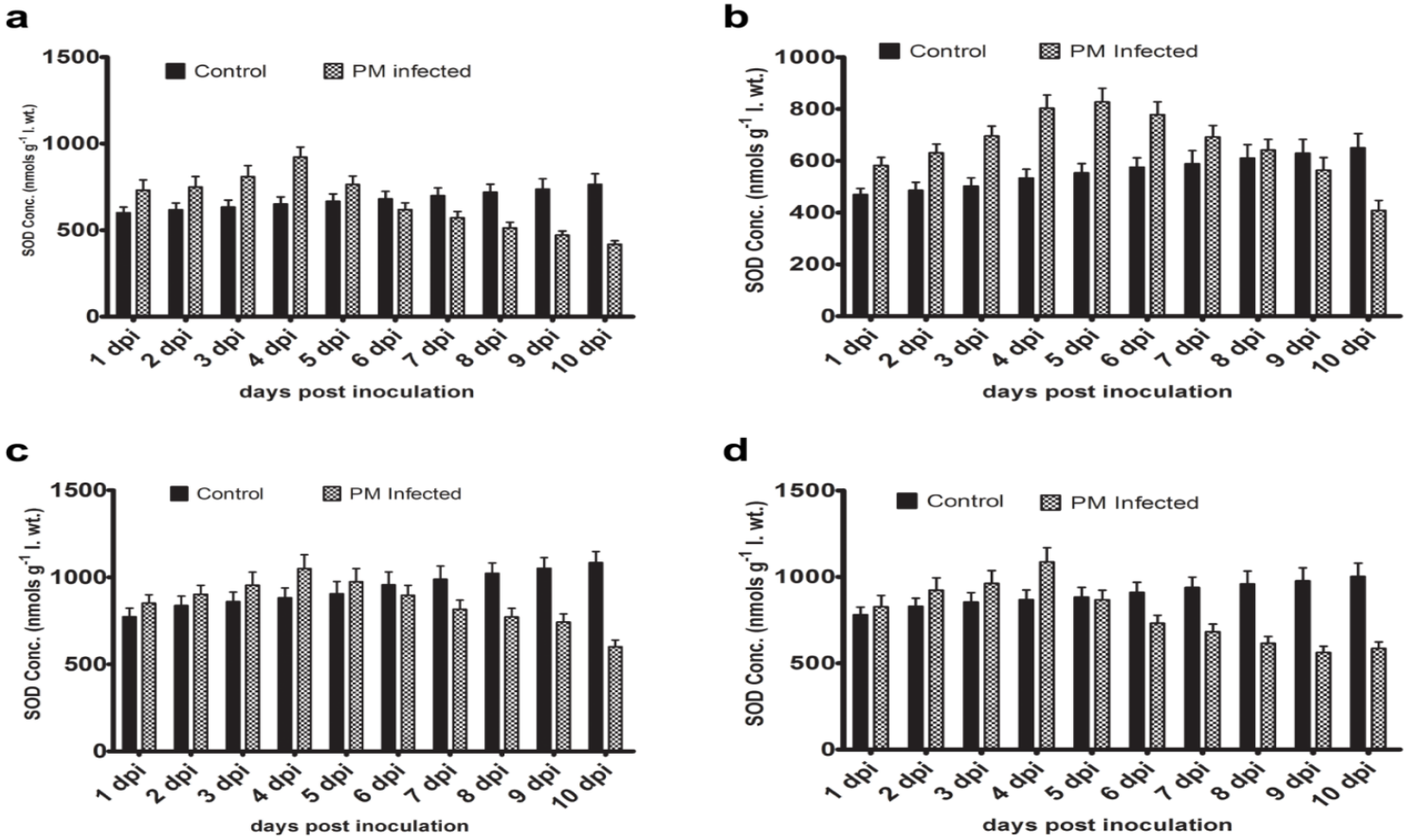

Fig. 5.13 Activities of SOD ((nmols /gram leaf. wt.)) in leaves of the susceptible (a-PS 2023 and b. Morden) and resistant (c-ID-25 and d-TX 16R) genotypes.

Table 5.3 Peroxidase **(**POX) Concentration (nmols/ gram leaf wt.)

Genotype	Condition	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
Morden	**Control**	118.95±2.26	124.5±2.3	130.42±0.21	128.33±0.2	130.38±0.21	133.81±0.95	136.06±0.96	141.24±1	150.16±1.06	152.5±1.08
	Infected	311.04±3.62	471.9±4.77	547.61±3.88	657.47±4.67	856.99±6.09	1095.47±7.78	847.78±5.31	425.71±2.31	114.83±0.1	90.99±0.06
PS 2023	**Control**	115.15±2.23	120.11±2.27	124.82±2.3	131.13±0.93	134.6±0.95	139.71±0.99	143.68±1.02	145.5±2.45	149.97±2.48	154.64±2.52
	Infected	420.87±4.41	917.57±7.94	954.42±6.78	1216.21±8.64	1548.56±11	1914.04±13.6	1457.33±9.64	1224.04±7.98	657.21±3.96	221.04±0.85
TX 16R	**Control**	101.48±0.01	104.21±0.02	109.56±0.06	111.51±0.79	117.22±0.83	121.8±0.86	126.28±0.89	127.78±2.32	132.59±2.36	141.12±2.42
	Infected	362.88±1.86	507.03±2.89	877.43±5.52	1096.81±7.79	1459.5±10.37	1133.2±8.05	906.9±6.44	860.92±7.53	681.44±6.26	404.08±4.29
ID-25	**Control**	244.48±1.02	250.72±1.07	258.9±1.83	262.99±1.86	268.78±1.91	272.08±1.93	274.85±3.37	279.34±3.4	288.41±3.47	293.99±3.51
	Infected	562.55±3.28	809.28±5.04	1206.46±8.57	1430.25±10.16	1749.99±12.43	1854.82±13.18	1420.38±10.09	918.08±7.94	715.75±6.5	536.24±5.23

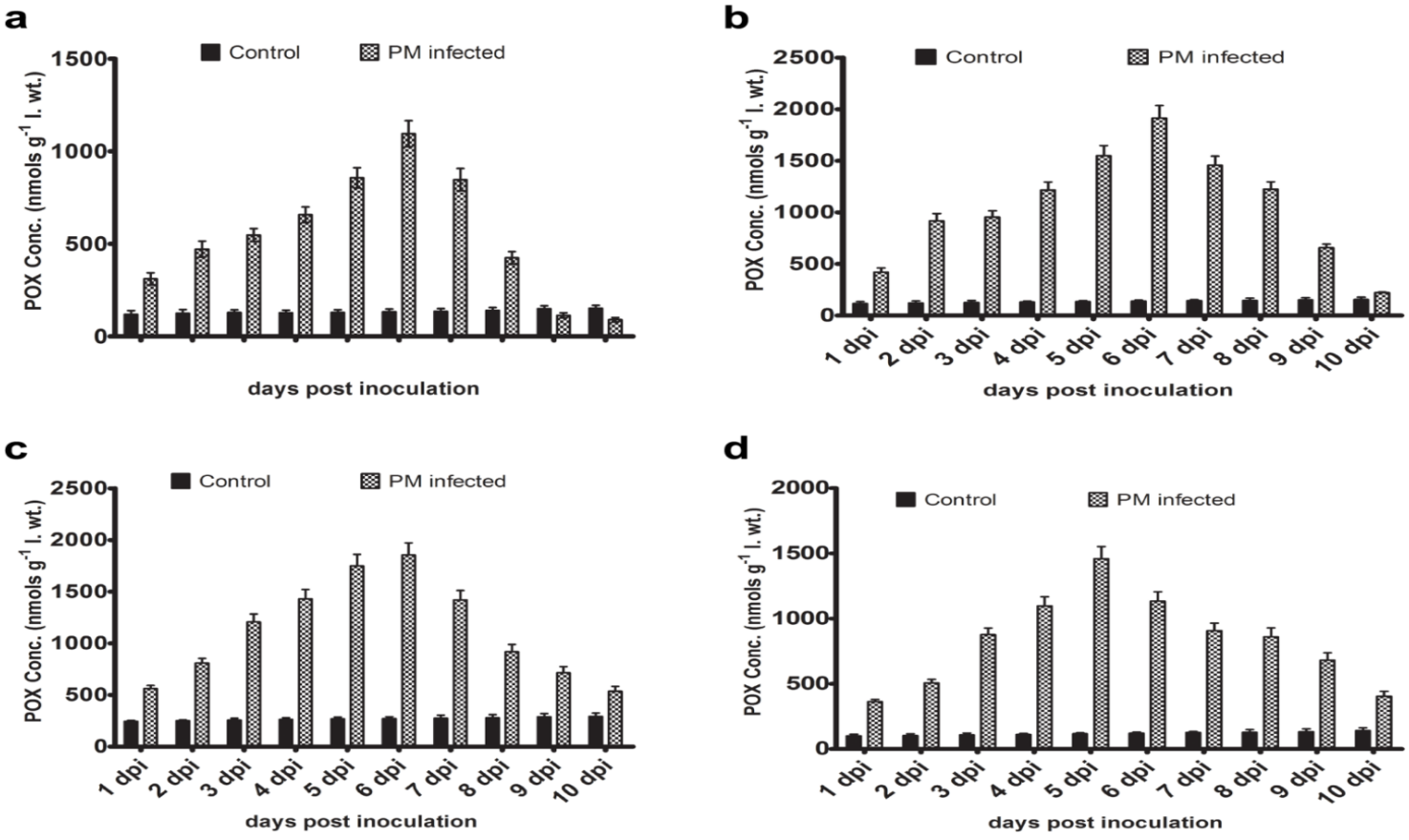

Fig. 5.14 Activities of POX ((nmols/ gram leaf wt.))in leaves of the susceptible (a-PS 2023 and b. Morden) and resistant (c-ID-25 and d-TX 16R) genotypes.

Table 5.4 Ascorbate peroxidase **(**APX) Concentration (nmols/ gram leaf. wt.)

Genotype	Condition	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
Morden	**Control**	141.62±1	157.77±1.12	170.56±1.21	182.83±1.29	197.49±1.4	209.62±2.91	232.61±3.07	254.54±3.23	277.24±3.39	289.01±3.47
	Infected	490.97±4.91	843.96±7.41	1102.97±9.25	1440.17±11.65	1622.79±11.53	1677.47±11.92	1707.29±12.13	1844.4±12.39	1872.04±12.59	1943.59±13.1
PS 2023	**Control**	156.05±1.1	160.47±1.14	167.25±1.19	173.41±1.23	188.12±0.62	202.34±0.72	231.18±0.92	236.84±3.1	239.97±3.12	249.21±3.19
	Infected	302.15±1.43	839.93±5.25	1018.87±8.66	1345.4±10.98	1619.42±12.92	1693.5±13.45	1949.19±15.27	1980.52±14.07	2030.27±14.42	2141.61±15.21
TX 16R	**Control**	171.75±1.22	189.52±1.34	205.31±1.45	245.77±3.17	258.8±3.25	275.38±3.37	288.33±3.46	310.76±3.62	330.66±3.76	346.2±3.88
	Infected	592.26±4.2	1053.43±7.48	1485.86±10.55	1817.05±12.91	2184.76±14.81	2485.63±16.95	2533.88±19.42	2723.4±20.77	2870.28±21.81	3199.58±24.15
	Control	210.42±2.91	235.68±3.09	246.47±3.17	254.9±1.81	260.95±1.85	266.82±1.89	271.84±1.93	277.67±1.26	288.95±1.33	313.21±1.51
ID-25	**Infected**	488.52±2.76	874.17±6.21	1204.26±8.56	1581.73±11.23	1859.49±14.63	2102.71±16.36	2237.74±17.32	2444.64±18.79	2503.87±19.21	2582.68±19.77

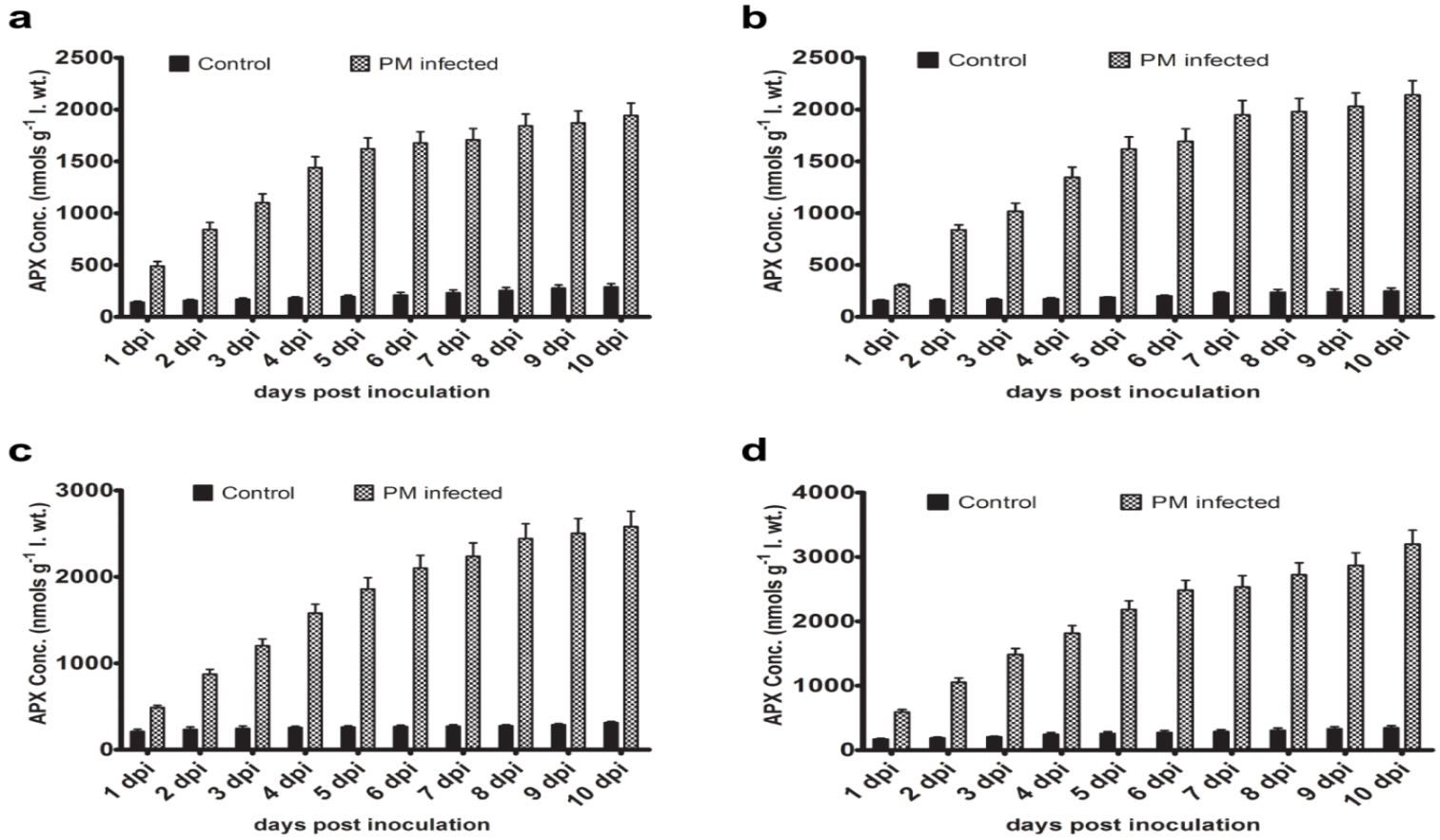

Fig. 5.15 Activities of APX ((nmols/ gram leaf. wt.)) in leaves of the susceptible (a-PS 2023 and b. Morden) and resistant (c-ID-25 and d-TX 16R) genotypes.

Table 5.5 Glutathione reductase (GR) Concentration (nmols/ gram leaf wt.)

Genotype	Condition	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	8 dpi	9 dpi	10 dpi
Morden	**Control**	2307.7±2.3	1870.47±2.3	1645.19±1.37	1536.08±2.3	1355.01±1.37	1197.7±2.3	926.25±1.37	837.79±2.3	567.87±1.37	422.32±1.4
	Infected	2576.35±1.4	2409.69±2.3	2133.9±1.4	2080.94±1.37	1792.69±1.4	1698.79±1.4	1325.38±1.37	1170.25±2.3	875.91±1.4	772.21±1.4
PS 2023	**Control**	2423.85±2.3	1986.17±1.37	1755.66±2.3	1676.78±1.4	1412.08±2.3	1220.36±1.4	1198.93±1.37	875.92±2.3	689.65±1.37	489.2±1.4
	Infected	2621.9±1.37	2526.7±1.4	2324.47±1.37	1991.38±2.3	1829.51±1.4	1684.63±2.3	1538.22±1.4	1377.01±1.37	848.68±1.4	527.34±1.37
TX 16R	**Control**	2793.78±2.3	2542.16±1.37	2462.41±2.3	2297.89±1.4	1901.84±2.3	1864.15±1.4	1517.54±1.37	1357.4±2.3	1197.28±1.37	995.52±1.4
	Infected	3090.47±2.3	2818.85±1.37	2624.94±2.3	2504.02±1.4	2460.85±2.3	2122.32±1.4	1964.28±1.37	1797.7±2.3	1606.08±1.37	1441.71±1.4
	Control	2568.51±2.3	2440.62±1.4	2186.81±2.3	1940.63±1.37	1757.62±2.3	1689.65±1.37	1417.58±1.4	1397.7±2.3	1151.51±1.4	847.59±1.4
ID-25	**Infected**	2882.45±2.3	26720.14±1.4	2519.05±2.3	2386.82±1.37	2289.86±2.3	2179.85±1.37	1916±1.4	1759.79±2.3	1504.67±1.4	1425.58±1.4

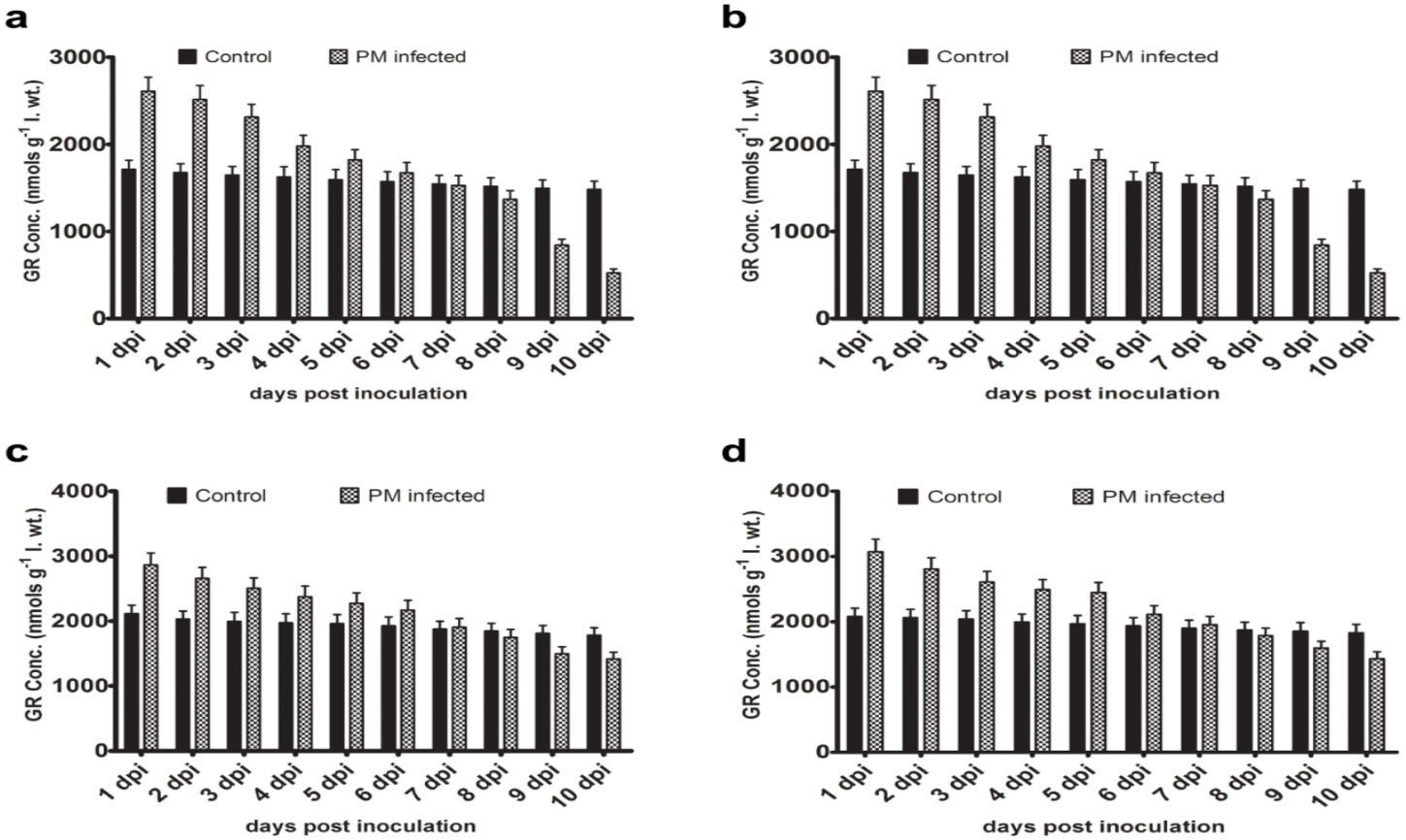

Fig. 5.16 Activities of GR (nmols/ gram leaf wt.) in leaves of the susceptible (a-PS 2023 and b. Morden) and resistant (c-ID-25 and d- TX 16R) genotypes.

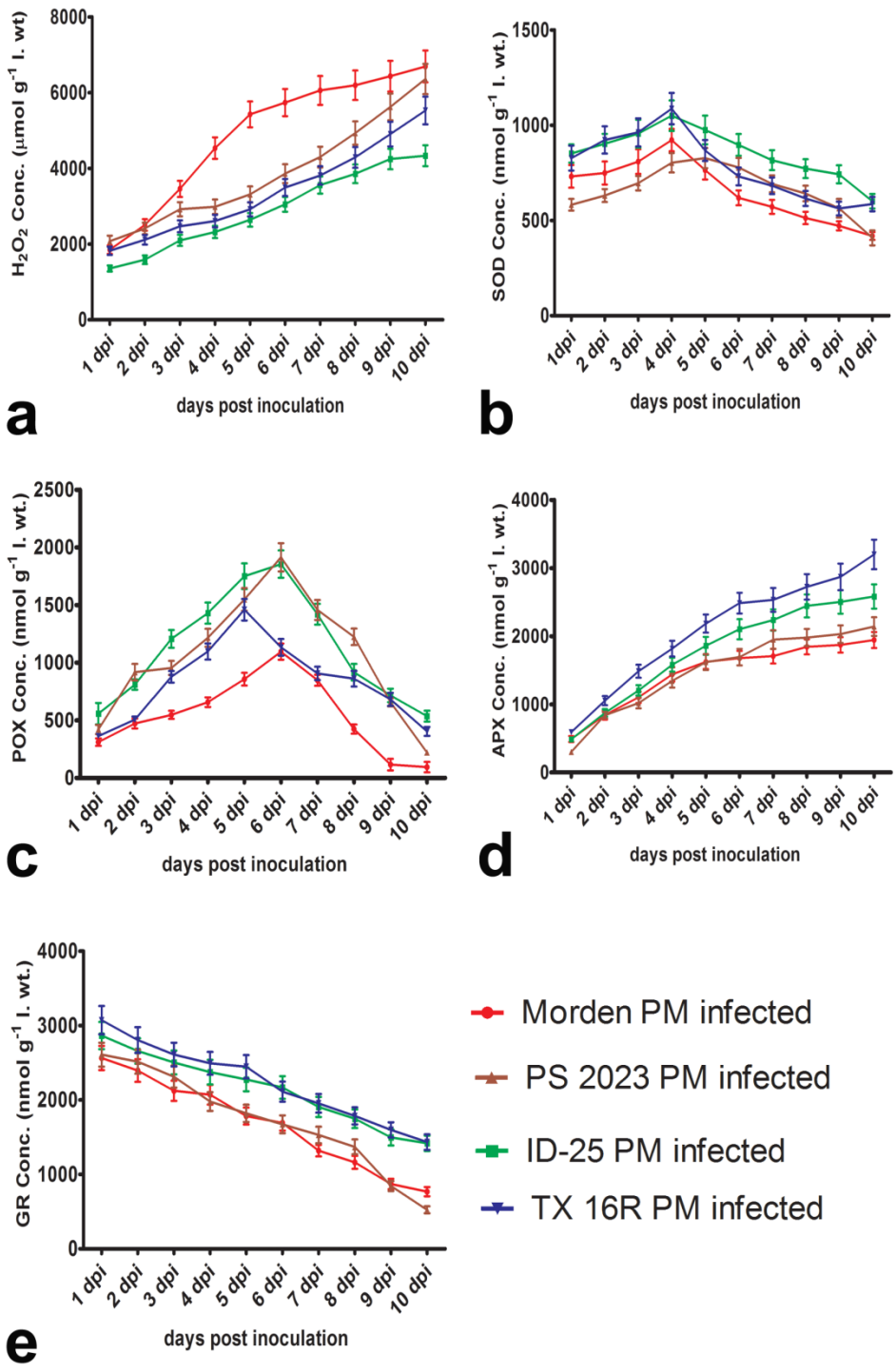

Fig. 5.17 Comparative graphs representing the activities of H_2O_2 (a), SOD (b), POX (c), APX (d) and GR (e) in leaves of the susceptible genotypes (PS 2023 and Morden) and resistant genotypes (ID-25 and TX 16R), from 1 to 10 dpi. H_2O_2- Hydrogen peroxide, SOD- Superoxide dismutase, POX-Peroxidase, APX- Ascorbate peroxide, GR- Glutathione reductase.

5.2. Discussion

Powdery mildews are considered to be best models for basic research on obligate parasite and plant interactions, developmental morphology, cytology, and molecular biology. However, recent research had shown the complexity in powdery mildews. There is great difficulty in identifying and characterization of powdery mildews due to their extensive diversity and their biology is more complex than generally realized (Yarwood, 1978; Braun et al., 2002). The combined use of classical morphological (light microscopy) and SEM analyses had successfully compared the morphological characteristics of powdery mildews (Kiss et al., 2001; Cook et al., 2006; 2011; Cook and Braun, 2009). In the present study, light and SEM analyses were carried out to examine the morphology and development of *G. orontii* on sunflower. To the best of our knowledge, this is the first attempt to compare the responses to the infection of the powdery mildew in resistant and susceptible sunflower genotypes in order to elucidate the infection process of *G. orontii* on sunflower.

The morphological features observed in the study are in agreement with the earlier studies (Cook et al., 1997; Cook and Braun, 2009), thus confirming the pathogens as *G. orontii*. Regardless of the growth and infectivity, the morphological features were the same in all the genotypes. Germination of conidia, formation of appressorium and haustorial complex in both susceptible and resistant genotypes at initial time points of infection indicated that *G. orontii* can establish a parasitic relationship with both the types of hosts regardless of the levels of resistance, but colony development and growth differed greatly depending on the level of tolerance. The fungal penetration into the host through stomata and junctions between epidermal cells was similar in both resistant (TX 16R and ID-25) and susceptible (PS 2023 and Morden) genotypes. However, the hyphal penetration through stomata was higher than the penetration through junctions of epidermal cells. Thus, the stomatal opening appears to play a key role in the penetration of germ tube and hyphae, whose opening is dependent on the relative humidity (RH).

Light microscopic observations showed that a diminished, late of development of *G. orontii* on resistant sunflower genotypes and reduced number of conidiophores per colony. The colony development and conidiophore formation of *G. orontii* on resistant genotypes appeared to be restricted after 4-5 dpi by the host defense mechanism(s). The number of sporulating conidiophores was reduced numbers in resistant genotypes, which exhibited

enhanced resistance. A significant reduction in fungal growth and development in resistant genotypes suggests that these genotypes had a defense mechanism against the *G. orontii* development. The defense induced in these genotypes is late but successful in preventing the later spread of sporulation of powdery mildew. Hence, although infection is visually evident on the lower leaves, it failed to spread to the upper leaves in the resistant genotypes. Arabidopsis mutants with enhanced disease resistance to powdery mildew were found to have similar reaction (Catherine and Roger, 1998; Vogel and Somerville, 2000). Thus, a distinct difference in fungal spread and host response were observed in susceptible and tolerant genotypes to powdery mildew.

The levels of accumulation of ROS species (O_2^- and H_2O_2) revealed distinct differences between the susceptible and resistant genotypes. In susceptible genotypes, the levels of ROS accumulation were high as evidenced by intense staining in tissues infected with the pathogen, whereas the levels of ROS in resistant genotypes were relatively low. The ROIs, particularly H_2O_2 prevent fungal penetration by two important mechanisms (Mellersh et al., 2002). In one model, ROIs acts as a signal for gene activation resulting in expression of antifungal biochemicals such as phenolics, which are secreted into plant cell wall. In the other model, H_2O_2 directly accounts for the inhibition of fungal penetration. The high reactivity of ROIs could be countered by the antioxidant mechanisms present in the host, failure of which results in loss of resistance (Breusegen et al., 2001). One of the key enzymes in antioxidant mechanisms of elimination of ROIs is SOD, which regulates the cellular concentration of O_2^- and H_2O_2. The bulk amount of H_2O_2 generated during the oxidative stress is eliminated by the cellular enzymes, catalase and peroxidase, whereas APX scavenges the H_2O_2 that is inaccessible for catalase, as APX has higher affinity to H_2O_2 (Breusegen et al., 2001).

The present study revealed that a relatively strong oxidative burst was induced in susceptible genotypes as compared to the resistant genotypes. The high level of accumulation of the ROIs triggers the activation of signal transduction thereby activating the signalling that results in up-regulation of the defense genes coding for PR-proteins, phytoalexin generating enzymes, lignification, oxidative stress protecting enzymes and other defense responses (Lamb and Dixon, 1997; Alvarez et al., 1998; Apel and Hirt, 2004). However, balanced state of ROIs is very important in triggering a hypersensitive response and other defense responses (Delledonne et al., 2001). The higher than threshold levels of

ROS would elicit a deleterious effect, which could be countered by the enzymatic and non enzymatic antioxidants (Asada and Takahashi, 1987; Bowler et al., 1991; Wilekens et al., 1997). The homeostasis of H_2O_2 plays a key role in plant defense (Wu et al., 1997; Levine et al., 1994). Thus, scavenging of ROS is very important in maintaining the homeostasis. The enzymatic scavenging mechanism is one of the important mechanisms that keep the lowest possible levels of ROS to prevent the host from oxidative stress damage.

In the present investigation, the levels of antioxidant enzymes like SOD, POX, APX and GR (key enzymes regulating oxidative burst) (Fig. 5.17) were studied in both susceptible (PS 2023 and Morden) and resistant (TX 16R and ID-25) genotypes against powdery mildew. A significant increase in concentration of SOD, POX, APX and GR was observed in genotypes subjected to powdery mildew infection than their respective controls. In comparison with susceptible genotypes, the levels of SOD, POX, APX and GR were higher in the resistant genotypes (Figure 5.17). This is the first report to assess the antioxidant enzyme regulation during powdery mildew infection in sunflower. Induced oxidative stress followed by a rise in the levels of SOD conferred resistance to powdery mildew in cucumber (Kang, 2008). The enhanced levels of SOD played a key role in imparting resistance against downy mildew disease in pearl millet. Transgenic expression of SOD conferred resistance to the fungus *C. betarcola* in sugarbeet (Tertivanidis et al., 2004). The increased levels of SOD conferred resistance to Fusarium wilt in chickpea (García-Limones et al., 2002). The downy mildew, a biotrophic fungus in sunflower provoked higher expression of SOD and GPX transcripts in incompatible sunflower genotypes (Herbette et al., 2003).

Peroxidases regulate biotic stress, either passively (building up of stronger walls through cross linking) or actively (production of ROS against the attacking pathogen). Peroxidases are reported to play a major role in the elimination of the pathogen (Passardi et al., 2004). The expression of an anionic peroxidase in the ripening tomato fruits by tissue specific expression under an ethylene inducible promoter E-8 resulted in fungal disease resistance (Divya et al, 2012). In the present work, enhanced levels of POX contributed significantly towards resistance exhibited by two resistant sunflower genotypes against powdery mildew. The high expression of POX in barley infected with powdery mildew induced phenolic depositions, resulting in defense against powdery mildew (Scot-Craig et al., 1995). The activity of peroxidase and catalase enzymes were also significantly increased in powdery mildew infected tolerant lines compared to the susceptible lines in flax (Ashry and

Mohamed, 2011). Elevated basal POX activity resulted in enhanced ability to arrest colonization of the fungus *Phoma mediaginin* in *Medicago truncatula* (Djebali et al., 2007). Rust resistant wheat leaves showed a significant increase in peroxidase activity during rust infection (Seevers et al., 1971). Similarly, downy mildew inoculated seedlings of pearl millet clearly recorded increased levels of peroxidase activity in highly resistant, resistant and induced resistant seedlings, where as susceptible and highly susceptible seedlings recorded decreases in the enzyme activity (Devaiah et al., 2003). Scopotein-dependent POX activity was induced only in resistant sunflower lines infected with downy mildew (Nandeeshkumar et al., 2008). Over production of APX in transgenic pepper increased the peroxidase activity that enhanced the ROS scavenging system, resulting in protection against oxidative stress induced by the Oomycetes (Sarowar et al., 2005). Transgenic Arabodpsis with a knockout of peroxidase showed an impaired oxidative burst and were more susceptible than the wild type plants to both bacterial and fungal pathogens (Bindschedler et al., 2006).

Apart from SOD and POX enzymes, the other key antioxidant enzymes that were involved in Asada-Halliwell pathway of hydrogen peroxide scavenging are APX and GR. The relative enhanced levels of these enzymes in resistant genotypes were in agreement with the earlier studies that reported the role of these enzymes in the regulation of oxidative stress induced by fungal pathogens and its useful role in imparting resistance (Aono et al., 1995; Venacker et al., 1998; Sarowar et al., 2005). Glutathione reductase acted as a resistance enhancer in *Haynaldia villosa* against powdery mildew (Chen et al., 2007). The reduced expression of ROI detoxifying enzymes APX and CAT in tobacco resulted in a hyper responsive phenotype against pathogen infection (Mittler et al., 1999). A pronounced activity of GPX, APX, CAT and SOD were detected in resistant sunflower lines compared with susceptible lines infected with *Sclerotinia sclerotiorum* (Davar et al., 2013).

The efficient scavenging of ROIs by antioxidant defense depends on the expression of ROI-scavenging enzymes. Higher ROIs production and low level expression of antioxidant enzymes resulted in increased ROIs accumulation in the susceptible genotypes. It is reported that excessive levels of ROIs lead to severe damage of the photosynthetic apparatus resulting in severe damage and chlorosis of the leaves along with membrane damage, which was observed in susceptible genotypes used in the present study. In resistant genotypes, higher production of ROI-scavenging enzymes was associated with protection to oxidative burst. Van Breusegem and Dat (2006) reported on the role of ROIs in plant signal

transduction and successfully demonstrated that ROIs induce defense genes and adaptive responses at low concentrations. In the present study, up-regulation of antioxidant enzymes maintains the homeostatic concentration of ROIs, which act as diffusible signals for induction of antioxidant and defense genes.

Finally, resistance to *G. orontii* in resistant genotypes is conferred at later stages in the infection. In both susceptible and resistant genotypes, *G. orontii* spores germinated and developed hyphae. Asexual reproduction was significantly reduced on resistant genotypes in terms of number of conidiophores as well as number of conidia. This indicates that resistance in resistant genotypes affected the fungal growth and spread primarily after the formation of conidiophores and reduced build up of spore load. Being sources of multiple resistance, these resistant accessions serve as promising donors in breeding for various resistance in sunflower in addition to powdery mildew.

Chapter 6

Differential proteomic analysis in sunflower - powdery mildew infection process

The present chapter reports on the study of sunflower- powdery mildew infection process in susceptible, resistant/tolerant and immune sunflower genotypes upon powdery mildew infection using proteomic approaches (Fig. 6.1) and results and discussion were presented in the following chapter

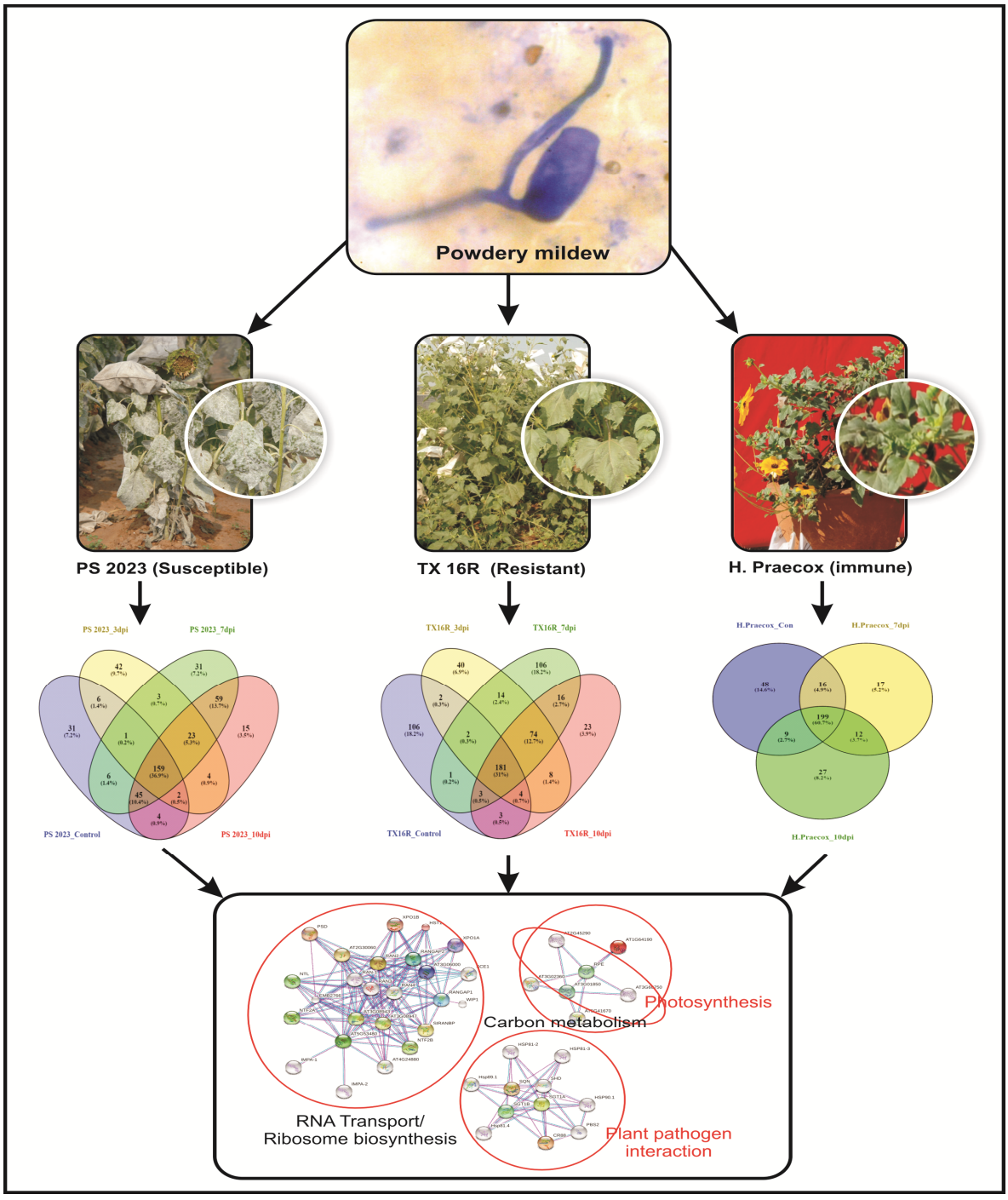

Fig. 6.1 Summary slide of the present investigation

6.1. Results

6.1.1. Light microscopy

Microscopic analysis of powdery mildew infection showed the initiation of infection process within 6 hours of inoculation with faster hyphal growth and penetration in the susceptible genotype (PS 2023) when compared with the resistant and immune genotypes in which the hyphal growth was sparse and observed only after 3 dpi (TX 16R) and 10 dpi (*H. praecox*) (Fig. 6.2). In the susceptible genotype (PS 2023), the hyphal growth of *G. orontii* increased rapidly and had maximum spread on the leaf within 7 dpi (Figs. 6.2 a, b, c), while as TX 16R (resistant) showed resistance to *G. orontii* penetration and allowed reduced hyphal expansion (Figs. 6.2 d, e, f) and the immune genotype (*H. praecox*) did not support hyphal development till 10 dpi (Figs. 6.2 g, h, i).

6.1.2. Quantitative analysis of total proteins

Number of proteins identified in the genotypes, PS 2023, TX 16R and *H. praecox* with quantification were 359, 413 and 306, respectively. Venn diagrams presented in Figure 5.3 show the common and differentially expressed proteins among quantified proteins in control and the tested time lines. In the susceptible genotype PS 2023, 159 (36.9%) proteins were common in the all three sampling time points including control. The number of proteins identified in 7 dpi and 10 dpi were higher than in PS 2023 control. In the resistant (TX 16R) genotype, a total of 181 (31.1%) proteins were common in all the three time points and control. The numbers of proteins identified at all three sampling time points were higher than in the control. In *H. praecox* (immune), there was no infection up to 7 days, thus only 7 dpi and 10 dpi were considered as sampling time points. A total of 199 (60.7%) proteins were common among the two sampling proteomes with control. The number of proteins identified at 7 dpi and 10 dpi were less compared to those in control (Fig. 6.3 a-c). Figure 5.3d-f shows the common and differentially expressed proteins among the quantified proteins in the three genotypes at control, 7 dpi and 10 dpi.

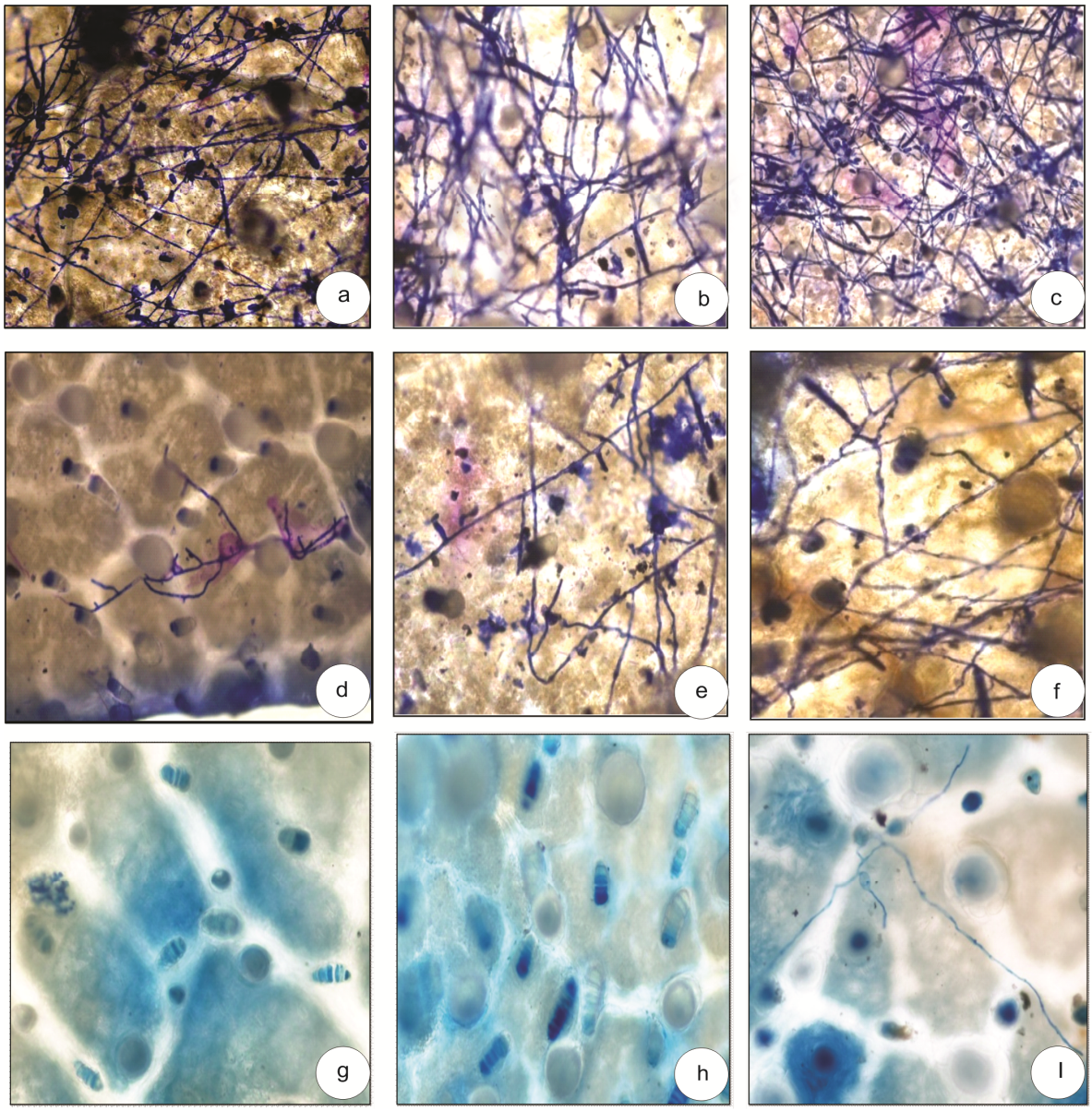

Fig.6.2. Light microscopic analysis of the development of *G. orontii* colony in susceptible (PS 2023) genotype at 3 dpi (a), 7 dpi (b), and 10 dpi (c), resistant (TX 16R) genotype at 3 dpi (d), 7 dpi (e), and 10 dpi (f)) and immune (*H. praecox)* genotype at 3 dpi (g), 7 dpi (h), and 10 dpi (i).

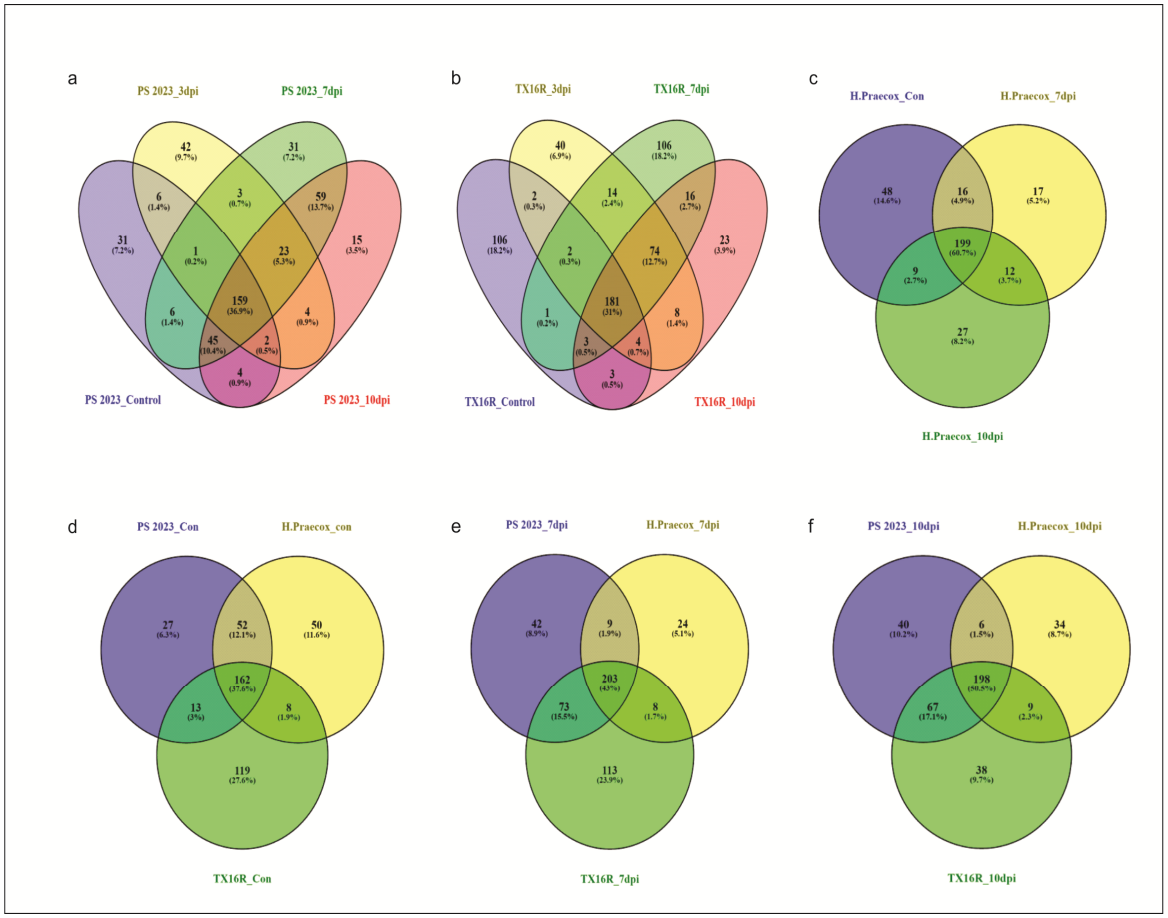

Fig. 6.3 Venn diagrams representing the number of identified proteins in the three genotypes at different time points. a) Control, 3 dpi, 7 dpi and 10 dpi of PS 2023 genotype b) Control, 3 dpi, 7 dpi, and 10 dpi, of TX 16R genotype c) Control, 3 dpi, 7 dpi and 10 dpi of *H. praecox* genotype d). Common proteomics among the three different genotypes at d) control, e) 7 dpi, f) 10 dpi.

6.1.3. Functional annotation and protein-protein interaction networks

Further, the possible mechanisms of regulation or functional pathways that are specifically powdery mildew-activated were studied. The molecular functional categorization at the three sampling time points in all three genotypes showed that the majority of proteins fell into catalytic processes followed by binding, structural and molecular activities (Fig. 6.4 a). The gene ontology of biological process for proteins of all three genotypes showed that the major categories of modulated proteins belong to metabolic processes followed by cellular processes and localization (Fig. 6.4 b). Cellular compartmentalization showed that 53.7% of the identified proteins belong to cell part followed by organelle (26.5%) and macromolecular complexs (16.9%) (Fig. 6.4 d). Classification of proteins showed that the majority of the proteins fell into oxidoreductase class proteins which play an important role in the modulation of oxidoreductase followed by proteins that belong to transferase, nucleic acid binding and stress response protein classes such as chaperons (Fig. 6.4 c).

The string 10.0 has generated the functional partnerships and interactions that occur between differentially expressed proteins of susceptible (PS 2023), resistant (TX 16R) and immune (*H. praecox*) genotypes. In the susceptible genotype, the protein-protein networks were carbon metabolism, sulfur metabolism, RNA transport and plant-pathogen interaction (Fig. 6.5 a). In the resistant genotype (TX 16R), the major networks were carbon metabolism, nitrogen metabolism, RNA transport, plant-pathogen interactions and photosynthesis (Fig. 6.5 b). In the immune (*H. praecox*) genotype, the major protein-protein interactions were carbon metabolism, nitrogen metabolism, photosynthesis and starch and sucrose metabolism (Fig. 6.5 c).

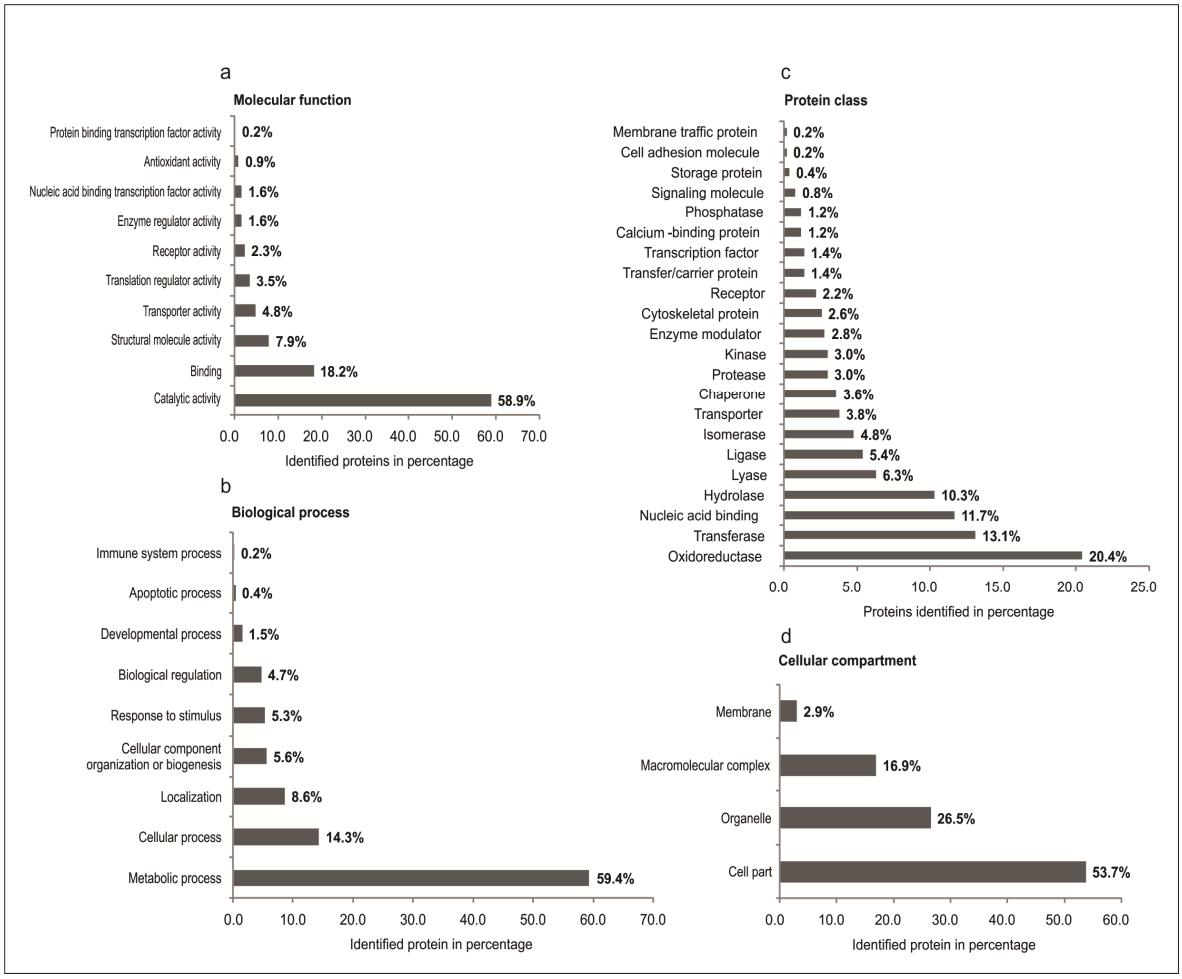

Fig. 6.4 Gene ontology (GO) based classification of the identified proteins in the three genotypes (PS 2023, TX 16R and *H. praecox*) by using PANTHER software tool. a) Molecular function, b) Biological function, c) Protein classification. d) Cellular components of the identified proteins.

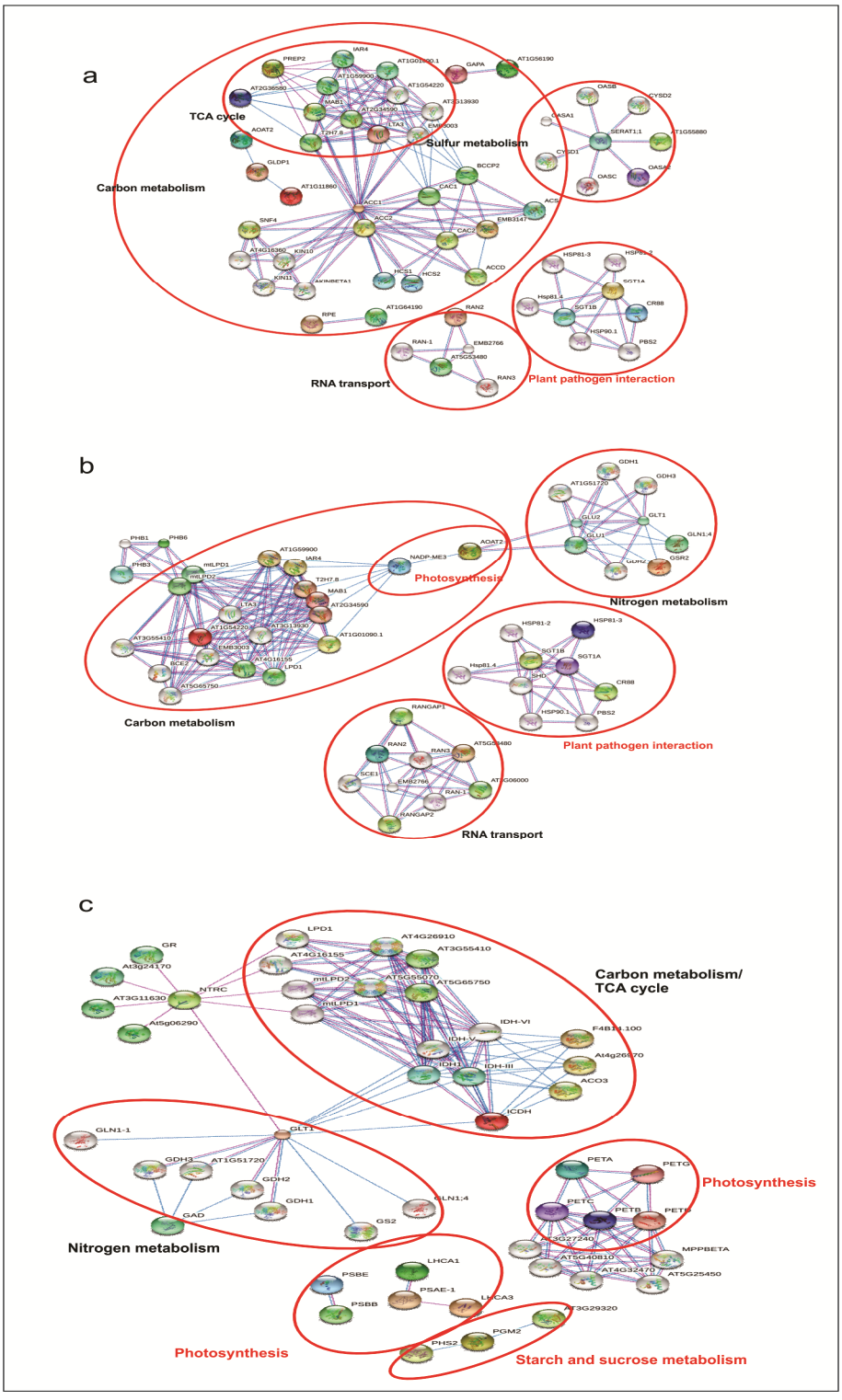

Fig. 6.5 Protein-protein interaction networks of differentially expressed proteins in sunflower genotypes in response to powdery mildew infection. a) Susceptible (PS 2023), b) Resistant (TX 16R), and c) Immune (*H. praecox*) genotypes.

6.1.4. Susceptible (PS 2023) proteome

Among the control and infected samples of the susceptible genotype (PS 2023) at all the 3 time points, a total of 42 differentially expressed proteins with a fold change threshold of 1.5 were selected (Table 6.1). Among these, a major group of 10 proteins (23.8%) belong to osmotic stress and second major group of 9 proteins are involved in photosynthesis (21.4%) followed by 9 proteins in response to chemical stimuli/inorganic substance (19%), 6 proteins of abiotic stress (14.2) and 3 proteins belonging to defense. Differentially expressed proteins in response to oxidative stress are 6-phosphogluconate dehydrogenase, catalase-2, CR88, ferritin-2, L-ascorbate peroxidase, malate dehydrogenase, pyridoxal biosynthesis, tubulin beta-8, UTP-glucose-1-phosphate uridylyl transferase and alanine-2-oxoglutarate aminotrnsferse-2. The upregulation of catalase-2 and L-ascorbic peroxidase proteins at 10 dpi, showed the evidence of oxidative stress in susceptible but the remaining proteins related to osmotic stress were shown to be regulated over the time points, which resulted in failure to prevent the oxidative stress generated by powdery mildew in the susceptible genotype. Photosynthesis related proteins includes chlorophyll a-b binding protein, glyceraldehyde 3-phosphate dehydrogenase, isoform long of ferredoxin-dependent glutamate, phosphoglycerate kinase, photosystem I reaction centre subunit, photosystem II CP-47 and photosystem Q. Most of these photosynthesis process related proteins were initially upregulated at 7 dpi and thereafter there was a downregulation at 10 dpi, which might have resulted in low level of photosynthesis in the susceptible genotype over the time points and resulted in low level of photosynthesis activity. Differentially expressed proteins related to defense are isocitrate dehydrogenase, isoform of carbonic anhydrous and peroxidoxin. There was a downregulation of two defense proteins *viz.,* isoform of carbonic anhydrous and peroxidoxin and low level expression of isocitrate dehydrogenase at 10 dpi.

Table 6.1 List of differentially expressed proteins in Susceptible genotype (PS 2023)

Protein IDs	Uniprot	Protein Descriptions	2023_ 3dpi Fold change	2023_ 8dpi	2023_ 10dpi	GOBP name	GOMF name	GOCC name
IPI00519417	Q9XFS9	1-deoxy-D-xylulose 5-phosphate reductoisomerase, chloroplastic	-0.13	1.23	1.54	Response to stimulus; small molecule metabolic process	1-deoxy-D-xylulose-5-phosphate reductoisomerase activity	Cell part; chloroplast
IPI00519564	Q9FWA3	6-phosphogluconate dehydrogenase family protein	1.58	0.74	0.95	Response to osmotic stress; response to salt stress; response to stimulus; response to stress	Phosphogluconate dehydrogenase (decarboxylating) activity	Cell part; chloroplast
IPI00516802	O04983	Acetyl-CoA carboxylase, biotin carboxylase subunit	0.03	1.92	1.03	Cellular biosynthetic process	Acetyl-coa carboxylase activity	Cell part; chloroplast
IPI00545323	Q9SF85	Adenosine kinase	-1	37.67	45.17	Adenosine biosynthetic process; response to stimulus	Adenosine kinase activity	Apoplast; cell part
IPI00538278	O65396	Aminomethyltransferase, mitochondrial	11.64	11.17	8.65	Response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Aminomethyltransferase activity	Apoplast; cell part
IPI00539634	B9DH91	AOAT2 (ALANINE-2-OXOGLUTARATE AMINOTRANSFERASE 2)	-1	3.89	1.48	Photorespiration; response to chemical stimulus; response to hypoxia; response to oxygen levels; response to stimulus; response to stress	1-aminocyclopropane-1-carboxylate synthase activity	Apoplast; cell part
IPI00523400	Q42080	ATB2; oxidoreductase	2.45	1.36	2.60	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Catalytic activity	Cell part; chloroplast
IPI00520641	P25819	Catalase-2	-1	3.68	4.11	Cell redox homeostasis; cellular response to hydrogen peroxide; cellular response to oxidative stress; response to stress	Antioxidant activity; peroxidase activity	Cell part; chloroplast; glyoxysome; intracellular membrane-bounded organelle
IPI00539195	Q8W585	Cell division protease ftsH homolog 8, chloroplastic	6.61	7.14	3.05	Cellular macromolecule metabolic process; developmental process	Adenyl nucleotide binding	Cell part; chloroplast
IPI00542715	Q07473	Chlorophyll a-b binding	-1	6.84	0.56		Chlorophyll binding; ion	Cell part; chloroplast

		protein CP29.1, chloroplastic					binding; metal ion binding; tetrapyrrole binding	
IPI00546413	Q8LPS0	CR88; ATP binding; CR88	6.24	2.52	3.07	Response to osmotic stress; response to stress; response to water; response to water deprivation	Adenyl nucleotide binding;	Cell part; chloroplast envelope;
IPI00529374	P47999	Cysteine synthase, chloroplastic/chromoplastic	5.02	6.22	5.15	Response to chemical stimulus; response to metal ion; response to stimulus;	Cysteine synthase activity	Apoplast; cell part
IPI00517335	Q56WX9	Elongation factor EF-2	1.10	1.15	1.96	Response to abiotic stimulus ; response to stress	Gtp binding; guanyl ribonucleotide binding	Cell junction; cell part;
IPI00520474	P17745	Elongation factor Tu, chloroplastic	3.36	2.04	1.57		GTP binding; guanyl nucleotide binding	Apoplast; cell part
IPI00547683	Q9LNP0	F1L3.35; GLYR2 (GLYOXYLATE REDUCTASE 2	-1	1.89	11.85	Carbohydrate catabolic process	Catalytic activity	Cell part; chloroplast part
IPI00517588	Q9SRL5	Ferritin-2, chloroplastic	4.49	-0.23	9.87	Biological regulation; cation transport; cellular cation homeostasis; cellular chemical homeostasis; response to oxidative stress; response to stimulus; response to stress	Catalytic activity; cation binding; ferric iron binding; oxidoreductase activity	Cell part; organelle; plastid
IPI00534642	Q9S7E4	Formate dehydrogenase, mitochondrial	3.54	2.05	4.39	Response to cadmium ion; response to chemical stimulus; response to stimulus	Catalytic activity; oxidoreductase activity	Cell part; chloroplast
IPI00542391	P55228	Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic	1.97	1.01	1.51	Glucan biosynthetic process; response to abiotic stimulus; response to stimulus; starch biosynthetic process	Adenyl nucleotide binding; adenylyltransferase activity; nucleotide binding; purine ribonucleoside triphosphate binding; transferase activity	ADPG pyrophosphorylase complex; apoplast; cell part
IPI00535907	Q9FMD9	Glutamine synthetase cytosolic isozyme 1-4	1.88	1.57	1.59	Glutamine biosynthetic process; glutamine family amino acid biosynthetic process	Glutamate-ammonia ligase activity	Cell part; cytoplasmic part
IPI00541680	P25857	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic	1.52	1.61	1.45	Photosynthesis, dark reaction; response to abiotic stimulus; response to cadmium ion; response to carbohydrate stimulus; response to chemical stimulus; response to cold; response to disaccharide stimulus; response to radiation; response to	Catalytic activity; coenzyme binding	Apoplast; cell part; chloroplast

						stress; response to temperature stimulus		
IPI00536025	Q94B78	Glycine dehydrogenase [decarboxylating] 2, mitochondrial	-1	4.52	2.41	Carboxylic acid catabolic process; response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Adenyl nucleotide binding; oxidoreductase activity	Apoplast; cell part; chloroplast part
IPI00535542	P41917	GTP-binding nuclear protein Ran-2	13.47	10.42	17.17	Cellular response to stimulus; establishment of localization in cell; intracellular protein transport; response to abiotic stimulus; response to chemical stimulus; response to inorganic substance; response to metal ion; response to osmotic stress; response to salt stress; response to stimulus; response to stress	Gtpase activity	Apoplast; cell junction;
IPI00539206	Q56ZW5	Isocitrate dehydrogenase, putative	0.46	0.95	1.76	Defense response; defense response to bacterium; response to abiotic stimulus; response to bacterium; response to biotic stimulus; response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to osmotic stress; response to other organism; response to salt stress; response to stimulus; response to stress; response to zinc ion	Catalytic activity; copper ion binding; isocitrate dehydrogenase (NADP+) activity; magnesium ion binding	Apoplast; cell junction; cell part
IPI00544626	P27140	Isoform 1 of Carbonic anhydrase, chloroplastic	-0.28	3.15	-0.16	Defense response; defense response to bacterium; defense response to fungus; defense response to fungus, incompatible interaction; defense response, incompatible interaction; immune response; innate immune response; multi-organism process; regulation of stomatal movement; response to abiotic stimulus; response to bacterium; response to biotic stimulus; response to cold; response to	Carbonate dehydratase activity	Apoplast; cell part; chloroplast envelope

						fungus; response to inorganic substance; response to stimulus; response to stress; response to temperature stimulus		
IPI00520267	O23404	Isoform 1 of Pyruvate, phosphate dikinase 1, chloroplastic	2.99	1.79	1.77	Photosynthesis	Pyruvate, phosphate dikinase activity	Cell part; chloroplast part
IPI00539225	Q9ZNZ7	Isoform Long of Ferredoxin-dependent glutamate synthase 1, chloroplastic	3.12	4.43	3.67	Photorespiration; response to abiotic stimulus; response to light stimulus; response to radiation; response to stimulus	Glutamate synthase activity; ion binding; oxidoreductase activity	Apoplast; cell part
IPI00520415	Q42564	L-ascorbate peroxidase 3, peroxisomal	-1	2.24	2.48	Cellular catabolic process; cellular cellular response to chemical stimulus; cellular response to hydrogen peroxide; cellular response to oxidative stress; cellular response to reactive oxygen species; cellular response to stimulus; cellular response to stress; response to stress	Antioxidant activity; catalytic activityr; peroxidase activity	Cell junction; cell part;
IPI00543463	P57106	Malate dehydrogenase, cytoplasmic 2	-1	2.22	1.30	Response to abiotic stimulus; response to chemical stimulus; response to inorganic substance; response to osmotic stress; response to salt stress; response to stimulus; response to stress	Catalytic activity; l-malate dehydrogenase activity; oxidoreductase activity	Apoplast; cell junction
IPI00521451	B2GVN3	OASA2 (O-ACETYLSERINE (THIOL) LYASE (OAS-TL) ISOFORM A1)	1.70	0.67	1.50	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus; response to zinc ion	Carbon-oxygen lyase activity; catalytic activity	Apoplast; cell part; chloroplast part
IPI00533612	Q949U7	Peroxiredoxin-2E, chloroplastic	-1	5.35	-1	Defense response; defense response to bacterium; response to bacterium; response to biotic stimulus; response to other organism; response to stimulus; response to stress	Antioxidant activity; catalytic activity; oxidoreductase activity, acting on peroxide as acceptor; peroxidase activity	Cell part; cell wall
IPI00530695	P50318	Phosphoglycerate kinase, chloroplastic	2.72	4.29	0.99	Photosynthesis, dark reaction; response to cadmium ion; response to chemical stimulus; response to	Phosphoglycerate kinase activity; phosphotransferase activity, carboxyl group as	Cell part; chloroplast part

						inorganic substance; response to metal ion; response to stimulus; small molecule catabolic process; small molecule metabolic process	acceptor	
IPI00546886	Q9S7N7	Photosystem I reaction center subunit V, chloroplastic	-1	1.60	1.53	Photosynthesis; regulation of photosynthesis; light reaction	Binding; chlorophyll binding; tetrapyrrole binding	Cell part; chloroplast part
IPI00529975	P56777	Photosystem II CP47 chlorophyll apoprotein	-0.51	2.43	0.28	Photosynthetic electron transport chain	Chlorophyll binding	Cell part; chloroplast part; photosystem
IPI00546934	P83755	Photosystem Q(B) protein	0.72	2.51	1.69	Photosynthetic electron transport chain; photosynthetic electron transport in photosystem II; response to chemical stimulus; response to herbicide; response to stimulus; response to stress; response to toxin	Electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity;	Cell part; chloroplast part
IPI00547030	Q9LJL3	Presequence protease 1, chloroplastic/mitochondrial	-1	3.49	-1	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Catalytic activity; hydrolase activity; ion binding; metal ion binding	Apoplast; cell part; chloroplast part
IPI00544908	Q8L940	Pyridoxal biosynthesis protein PDX1.3	1.76	1.36	0.98	Hyperosmotic response; hyperosmotic salinity response; response to abiotic stimulus; response to chemical stimulus	Binding; catalytic activity; identical protein binding	Cell part; chloroplast
IPI00519778	Q94KE3	pyruvate kinase, putative	1.84	0.34	-0.01	Alcohol catabolic process; response to cadmium ion; response to chemical stimulus; response to stimulus	Pyruvate kinase activity;	Cell part; cytoplasmic part
IPI00546385	C0Z3J9	RPE; catalytic/ ribulose-phosphate 3-epimerase	-1	2.68	-0.55	Response to abiotic stimulus; response to biotic stimulus; response to cold; response to nematode; response to other organism; response to stimulus; response to stress; response to temperature stimulus	Racemase and epimerase activity	Apoplast; cell part
IPI00524792	Q93VC7	RPS1 (RIBOSOMAL PROTEIN S1	0.29	1.94	0.48		RNA binding	Cell part; chloroplast part
IPI00528567	P48578	Serine/threonine-protein phosphatase PP2A-3 catalytic subunit	-1	1.80	1.40	Protein modification process	Catalytic activity; hydrolase activity; acting on ester bonds	Cell part; cytoplasm

IPI00539093	P29516	Tubulin beta-8 chain	3.49	2.40	2.05	Cellular component organization or biogenesis; response to abiotic stimulus; response to osmotic stress; response to salt stress; response to stimulus; response to stress	Gtpase activity; guanyl nucleotide binding; guanyl ribonucleotide	Cell part; cytoplasmic part
IPI00537899	P57751	UTP--glucose-1-phosphate uridylyltransferase 1	2.12	2.00	1.52	Callose deposition in cell wall; response to external stimulus; cellular response to starvation; cellular response to stress; response to abiotic stimulus; response to chemical stimulus; response to external stimulus; response to osmotic stress; response to salt stress; response to stress	Catalytic activity; utp:glucose-1-phosphate uridylyltransferase activity	Cell part; cell projection

***The multiple function of the same protein is given in the tables**

6.1.5. Resistant (TX 16R) proteome

A total of 32 differentially expressed proteins with a fold change threshold of 1.5 were selected in TX 16R (Table 6.2). A major group of differentially expressed proteins that responded to powdery mildew infection were those involved in response to oxidative stress, which includes alanine-2-oxoglutarate aminotransferase-2, catalase-2, CR88 and glutamine synthetase. The upregulation of proteins, CR88 and glutamine synthetase might have resulted in maintenance of homeostatis of the host cell by regulating the levels of reactive oxygen intermediates generated in the host following pathogen attack. Proteins involved in photosynthesis were photosystem II CP-47, Psbp domain containing protein and Tubulin beta-8 chain. The upregulation of all these three photosynthesis related proteins at 10 dpi possibly enhanced the rate of photosynthesis and helped the resistant genotype in withstanding the starvation that resulted as a consequence of pathogen attack. The defense related proteins expressed in resistant were heat shock cognate 70 kDa protein-1 and heat shock protein 81-2. The abundance of heat shock proteins was absent/very low in susceptible genotype (PS 2023).

Table 6.2 List of differentially expressed proteins in Resistant genotype (TX 16R)

Protein ID	Uniprot ID	Protein Descriptions	TX 16R (3 dpi)	TX 16R (8 dpi)	TX 16R (10 dpi)	*GOBP name	*GOMF name	*GOCC name
IPI00546269	P51427	40S ribosomal protein S5-2	0.54	4.66	3.04	Biosynthetic process; cellular biosynthetic process	Structural constituent of ribosome	Cell junction; cell part; cell wall
IPI00545955	Q8LG34	6-phosphogluconate dehydrogenase family protein	1.81	2.28	2.90	Hexose catabolic process; hexose metabolic process	Catalytic activity; oxidoreductase activity	Cell junction; cell part
IPI00535813	P10671	Actin-1/3	-0.38	-0.64	2.93		Adenyl nucleotide binding	Cell part; cytoplasm
IPI00531318	P52901	ALPHA Pyruvate dehydrogenase E1 component subunit alpha-1, mitochondrial	-0.36	1.48	0.54	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	pyruvate dehydrogenase (acetyl-transferring) activity	Cell part; cytoplasmic part
IPI00539634	B9DH91	AOAT2 (ALANINE-2-OXOGLUTARATE AMINOTRANSFERASE 2)	3.16	0.68	1.47	Response to chemical stimulus; response to hypoxia; response to oxygen levels; response to stress	Alanine-glyoxylate transaminase activity	Apoplast; cell part; chloroplast; peroxisome; plastid
IPI00518644	B2CT24	APS1 (ATP SULFURYLASE 1)	1.41	1.05	1.76	Cellular response to external stimulus; cellular response to nutrient levels; cellular response to starvation; cellular response to stimulus; cellular response to stress; cellular response to sulfate starvation; response to cadmium ion; response to chemical stimulus;	Adenyl nucleotide binding; adenyl ribonucleotide binding; adenylyltransferase activity	Cell part; chloroplast part; chloroplast

						response to inorganic substance; response to stress		
IPI00527702	Q9SZX3	Argininosuccinate synthase	1.28	0.94	2.74	Arginine metabolic process; cellular amine metabolic process; cellular nitrogen compound biosynthetic process; small molecule metabolic process	Argininosuccinate synthase activity;	Cell part; chloroplast part
IPI00523400	Q42080	ATB2; oxidoreductase	0.98	1.95	5.91	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Catalytic activity; oxidoreductase activity	Cell part; cytoplasmic part
IPI00535942	Q9XGZ0	ATNADP-ME3 (NADP-malic enzyme 3)	0.93	1.17	1.65	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to stimulus	Malate dehydrogenase (oxaloacetate-decarboxylating) activity; oxidoreductase activity	Apoplast; cell junction; cell part
IPI00542196	Q9SIL6	ATPHB6	2.18	-0.00	0.84	Response to stimulus; response to stress		Cell part; cell wall; chloroplast; cytoplasmic part; external encapsulating structre
IPI00520641	P25819	Catalase-2	1.56	4.25	4.08	Cell death; cell redox homeostasis; cellular homeostasis; cellular response to chemical stimulus; cellular response to external stimulus; cellular response to hydrogen peroxide; cellular response to nitrogen starvation; cellular	Antioxidant activity; catalase activity; oxidoreductase activity; oxidoreductase activity, acting on peroxide as acceptor; peroxidase activity	Cell part; chloroplast; cytoplasmic part; cytosolic part; peroxisome; plastid

						response to oxidative stress; cellular response to phosphate starvation; cellular response to reactive oxygen species; cellular response to starvation; cellular response to stimulus; cellular response to stress; death; homeostatic process; hydrogen peroxide catabolic process; hydrogen peroxide; reactive oxygen species metabolic process; response to abiotic stimulus; response to chemical stimulus; response to cold; response to extracellular stimulus; response to hydrogen peroxide; response to oxidative stress; response to reactive oxygen species; response to stimulus; response to temperature stimulus		
IPI00543476	Q9LZF6	Cell division control protein 48 homolog E	0.67	1.17	2.04	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Adenyl nucleotide binding	Cell junction; cell part; cell wall
IPI00519439	Q9LR75	Coproporphyrinogen-III oxidase	0.41	1.42	2.06	Biosynthetic process; cellular biosynthetic	Coproporphyrinogen oxidase activity;	Apoplast; cell part; chloroplast part

						process	oxidoreductase activity	
IPI00546413	Q8LPS0	CR88; ATP binding	9.49	4.14	5.36	Response to abiotic stimulus; response to chemical stimulus; response to chlorate; response to heat; response to inorganic substance; response to light stimulus; response to osmotic stress; response to radiation; response to salt stress; response to stimulus; response to stress; response to temperature stimulus; response to water; response to water deprivation	Adenyl nucleotide binding	Cell part; chloroplast envelope
IPI00938969	Q9LFN6	DEAD/DEAH box helicase, putative	0.20	0.18	2.58	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Adenyl nucleotide binding	Cell junction; cell part; cell wall
IPI00534642	Q9S7E4	Formate dehydrogenase, mitochondrial	2.33	3.93	4.97	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Coenzyme binding; formate dehydrogenase	Cell part; chloroplast
IPI00535907	Q9FMD9	Glutamine synthetase cytosolic isozyme 1-4	0.90	2.01	3.66	Amine biosynthetic process	Acid-ammonia (or amide) ligase activity	Cell part; cytoplasmic part
IPI00534852	Q43127	Glutamine synthetase, chloroplastic/mitochondrial	3.29	1.40	1.20	Aging; amine biosynthetic process; response to abiotic	Acid-ammonia (or amide) ligase activity	Apoplast; cell part

						stimulus; response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to osmotic stress; response to salt stress; response to stimulus; response to stress		
IPI00535542	P41917	GTP-binding nuclear protein Ran-2	1.13	1.61	3.81	Cellular response to stimulus; response to abiotic stimulus; response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to osmotic stress; response to salt stress; response to stimulus; response to stress	Gtpase activity	Apoplast; cell junction; cell part
IPI00530209	Q8LPQ4	HDS (4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE SYNTHASE)	1.66	1.83	4.51	Cellular biosynthetic process	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase activity; binding	
IPI00543293	P22953	Heat shock cognate 70 kDa protein 1	2.52	1.20	2.24	Defense response; defense response to bacterium; defense response to fungus; response to biotic stimulus; response to cadmium ion; response to chemical stimulus; response to cold; response to fungus;	Adenyl nucleotide binding	Apoplast; cell junction; cell part

						response to heat; response to inorganic substance; response to metal ion; response to stimulus; response to stress; response to temperature stimulus; response to virus; stomatal closure; stomatal movement		
IPI00537112	P55737	Heat shock protein 81-2	0.15	2.52	-0.60	Cellular response to calcium ion; cellular response to chemical stimulus; cellular response to inorganic substance; defense response to bacterium; defense response to bacterium, incompatible interaction; defense response, incompatible interactionimmune response ; immune system process; innate immune response; response to abiotic stimulus; response to biotic stimulus; response to heat; response to osmotic stress; response to salt stress; response to stimulus; response to stress; response to temperature stimulus; response to water deprivation; stomatal movement	Adenyl nucleotide binding; catalytic activity; hydrolase activity	Apoplast; cell junction; cell part; external encapsulating structure
IPI00521247	O23629	Histone H2B.6	-0.87	2.21	-0.80	Cellular component	Nucleic acid binding;	Cell junction; cell part

						assembly at cellular level; protein-DNA complex subunit organization	protein binding	
IPI00520838	P59259	Histone H4	-0.50	4.76	-0.68	Chromosome organization; response to abiotic stimulus; response to chemical stimulus; response to stimulus; response to stress; response to water; response to water deprivation	Nucleic acid binding; protein binding	Cell junction; cell part
IPI00530695	P50318	Phosphoglycerate kinase, chloroplastic	3.70	1.79	2.64	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus;	Kinase activity; transferase activity	Cell part; chloroplast part
IPI00529975	P56777	Photosystem II CP47 chlorophyll apoprotein	4.68	2.29	2.57	Cellular macromolecule metabolic process;	Chlorophyll binding	Cell part; chloroplast part
IPI00541448	Q944G9	fructose-bisphosphate aldolase, putative	1.76	0.31	-0.00	Response to cadmium ion; response to chemical stimulus; response to endogenous stimulus; response to inorganic substance; response to organic substance	Catalytic activity; fructose-bisphosphate aldolase activity	Apoplast; cell part; chloroplast envelope
IPI00546705	Q9ZU25	mitochondrial processing peptidase alpha subunit, putative	2.08	0.98	1.32	Response to abiotic stimulus; response to osmotic stress; response to salt stress;; response to stress	Catalytic activity; endopeptidase activity; hydrolase activity	Cell part; chloroplast; cytoplasmic part; vacuolar part
IPI00516840	Q9LXX5	PsbP domain-containing protein 6, chloroplastic	3.67	1.41	3.30	Cellular metabolic process; metabolic process; photosynthesis	Calcium ion binding; protein binding	Cell part; chloroplast part

IPI00542147	Q9FKQ3	Putative histone H3-like 5	-0.87	2.30	-0.89	Cellular component assembly; cellular component assembly at cellular level	Nucleic acid binding; protein binding	Cell part; chromosomal part
IPI00540396	Q56YA5	Serine--glyoxylate aminotransferase	5.74	1.97	2.65	Biosynthetic process; cellular biosynthetic process; photorespiration	Alanine-glyoxylate transaminase activity; catalytic activity	Apoplast; cell part; chloroplast part; peroxisome; plastid part
IPI00539093	P29516	Tubulin beta-8 chain	1.73	0.55	0.97	Cellular component assembly; response to abiotic stimulus; response to osmotic stress; response to salt stress	Binding; catalytic activity; gtpase activity	Cell part; cytoplasmic part; golgi apparatus; intracellular organelle

*** The multiple function of the same protein was given in the tables**

6.1.6. Immune (*H. praecox*) proteome

Among the control and infected samples, a total of 16 proteins differentially expressed with a fold change threshold of 1.5 were selected (Table 6.3). The major group of differential proteins that responded to powdery mildew infection included those proteins that are involved in photosynthesis. The proteins associated with photosynthesis were apocytochrome-f, chlorophyll a-b binding, cytochrome b559 subunit alpha, LHCA-1, LHCA-3, LHCB-3, photosystem I iron-sulfur center, ATRAB7B and photosystem-I. Upon observing the expression patterns over the two sampling time points in comparison with the control, nine proteins were upregulated at both 7 dpi and 10 dpi whereas, 7 were upregulated at 7 dpi and then downregulated at 10 dpi. The initial upregulation of a few photosynthesis proteins at 7 dpi and then downregulation at 10 dpi might be because of initial pathogen attack and subsequent failure of the pathogen to invade the immune genotype. The only protein expressed in response to oxidative stress was NADPH-dependent thioredoxin reductase-C.

Table 6.3 List of differentially expressed proteins in Immune genotype (*H. praecox*)

Protein IDs	Uniprot	Protein Descriptions	*H. praecox* 8 dpi	*H. praecox* 10 dpi	*GOBP name	*GOMF name	*GOCC name
IPI00537792	P40941	ADP,ATP carrier protein 2	0.74	2.41	Cellular process; establishment of localization	Active transmembrane transporter activity; antiporter activity; atp:adp antiporter activity; cation binding	Cell part; cell wall
IPI00547341	P56771	Apocytochrome f	0.33	1.73	Cellular metabolic process; oxidation-reduction process; photosynthesis; transport	Cation binding; heme binding; metal ion binding; tetrapyrrole binding; transition metal ion binding	Cell part; chloroplast part
IPI00537989	Q42248	ATRAB7B (ARABIDOPSIS RAB GTPASE HOMOLOG G3F)	0.48	2.25	Biological regulation; cellular response to stimulus; response to abiotic stimulus; response to chemical stimulus; response to osmotic stress; response to oxidative stress; response to salt stress; response to stimulus; response to stress	GTP binding; guanyl ribonucleotide binding	Cell part; cytoplasmic part
IPI00527705	P04777	Chlorophyll a-b binding protein 165/180	0.54	1.61	Photosynthesis, light harvesting; response to abiotic stimulus; response to blue light; response to far red light; response to light stimulus; response to radiation; response to red light; response to red or far red light; response to stimulus	Chlorophyll binding; metal ion binding; tetrapyrrole binding	Cell part; chloroplast
IPI00520987	P56779	Cytochrome b559 subunit alpha	2.19	0.75	Oxidation-reduction process; photosynthesis		Cell part; chloroplast

IPI00534642	Q9S7E4	Formate dehydrogenase, mitochondrial	3.62	2.09	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Formate dehydrogenase (nad+) activity; oxidoreductase activity; oxidoreductase activity	
IPI00530557	Q42521	Glutamate decarboxylase 1	0.63	2.06	Response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Pyridoxal phosphate binding; vitamin b6 binding	Cell junction; cell part
IPI00530201	Q945K7	Isocitrate dehydrogenase [NAD] catalytic subunit 5, mitochondrial	2.66	0.21	Acetyl-coa metabolic process; carboxylic acid metabolic process	Isocitrate dehydrogenase (NAD+) activity; oxidoreductase activity	Cell part; chloroplast
IPI00516813	B9DHK2	LHCA1	1.92	0.41	Photosynthesis, light harvesting; response to abiotic stimulus; response to light stimulus; response to radiation; response to red or far red light; response to stimulus	Cation binding; chlorophyll binding	Cell part; chloroplast
IPI00532189	Q0WR83	LHCA3; chlorophyll binding	2.48	1.66	Photosynthesis, light harvesting	Structural molecule activity	Cell part; chloroplast
IPI00532626	C0Z239	LHCB3 (LIGHT-HARVESTING CHLOROPHYLL B-BINDING PROTEIN 3); structural molecule	1.11	1.93	Photosynthesis, light harvesting	Structural molecule activity	Cell part; chloroplast

IPI00526843	Q9SCY0	Phosphoglucomutase, chloroplastic	-0.20	2.65	; Detection of abiotic stimulus; detection of external stimulus; detection of stimulus; response to abiotic stimulus; response to cold; response to external stimulus; response to gravity; response to stimulus; response to stress; response to temperature stimulus	Catalytic activity; intramolecular transferase activity; ion binding	Apoplast; cell part
IPI00541194	P62090	Photosystem I iron-sulfur center	0.74	2.54	Photosynthetic electron transport chain	Oxidoreductase activity	Cell part; chloroplast part
IPI00546886	Q9S7N7	Photosystem I reaction center subunit V, chloroplastic	1.49	0.42	Biological regulation; oxidation-reduction process; photosynthesis; photosynthetic electron transport chain; photosystem I stabilization	Chlorophyll binding; tetrapyrrole binding	Apoplast; cell part
IPI00547030	Q9LJL3	Presequence protease 1, chloroplastic/mitochondrial	4.52	4.37	Proteolysis; response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Catalytic activity; endopeptidase activity; hydrolase activity; peptidase activity	Apoplast; cell part

IPI00529027	O22229	NTRC (NADPH-DEPENDENT THIOREDOXIN REDUCTASE C)	1.09	3.80	Cell redox homeostasis; cellular response to chemical stimulus; cellular response to hydrogen peroxide; cellular response to oxidative stress; cellular response to oxygen radical; cellular response to reactive oxygen species; cellular response to stimulus; cellular response to stress; cellular response to superoxide; homeostatic process; oxidation-reduction process; response to hydrogen peroxide; response to inorganic substance; response to oxygen radical; response to reactive oxygen species; response to stimulus;	Antioxidant activity; catalytic activity; enzyme activator activity; oxidoreductase activity; oxidoreductase activity	Cell part; chloroplast part

***The multiple function of the same protein is given in the tables**

6.1.7. Differentially expressed proteins common in both susceptible and resistant genotypes

Eight proteins were commonly expressed in both the susceptible (PS 2023) and resistant (TX 16R) genotypes in response to pathogen attack and include 6-phosphogluconate dehydrogenase, oxidoreductase, ATP binding, formate dehydrogenase, catalase, GTP-binding nuclear protein Ran-2, alanine-2-oxoglutarate aminotransferase 2 and photosystem II CP 47 (Fig. 6.6). All these proteins with the exception of photosystem II CP 47 are related to those that are expressed in response to stress, while photosystem II CP 47 acts in photosynthesis. The level of expression of these proteins was more in resistant (TX 16R) than the susceptible (PS 2023) genotype. In case of GTP-binding protein Ran-2, the level of expression was more in susceptible than the resistant genotype (Fig. 6.6). Formate dehydrogenase was the only stress protein that was expressed in all the three genotypes and the level of expression was more in immune (*H. praecox*) than the other two genotypes (Table 6.4).

6.1.8. Validation of differentially expressed proteins by analysis of gene expression by qRT-PCR

Nine differentially expressed proteins were selected for validation through qRT-PCR. A comparative expression levels of these genes in both susceptible (PS 2023) and resistant (TX 16R) genotypes in response to powdery mildew attack at similar time points (3 dpi, 7 dpi and 10 dpi) were represented in figure 6.7. The expression levels of Catalase and ATP-sulfurylase, which were belongs to oxidative stress were up regulated in resistant compared to the susceptible genotype. The proteins, 6-phosphogluconate dehydrogenase, Pyruvate dehydrogenase and Glutamine synthetase were the key enzyme involved in the carbon metabolic process and play a pivotal role in oxidative stress. These proteins were up-regulated in all time points in resistant, whereas they were down-regulated in susceptible genotypes. The results were in agreement with the protein levels expressed in these genotypes. But in case of Formate dehydrogenase, another oxidative stress protein, it was up-regulated up to 3 dpi in resistant and then it was down-regulated at 7 dpi and 10 dpi in comparison with susceptible genotype. The defence genes like HSP (HSP-70) and Heat shock transcription factor were strongly expressed in resistant (TX 16R) in all time points, whereas in susceptible (PS 2023) they were expressed in low levels. These results were in co-ordination with the protein levels expressed. Similarly, the protein LHC, which belongs to photosynthesis was up-regulated in resistant in comparison with susceptible.

Table 6.4 List of differentially expressed proteins common in Susceptible (PS 2023), Resistant (TX 16R) and Immune (*H. praecox*) genotypes.

Protein ID	Uniprot ID	Protein Descriptions	*GOBP name	*GOMF name	*GOCC name
IPI00545955	Q8LG34	6-phosphogluconate dehydrogenase family protein	Hexose metabolic process	Catalytic activity; oxidoreductase activity	Cell junction; cell part
IPI00539634	B9DH91	Alanine-2-Oxoglutarate Aminotransferase 2	Response to hypoxia; response to oxygen levels; response to stress	Alanine-glyoxylate transaminase activity	Apoplast; cell part; chloroplast; peroxisome; plastid
IPI00523400	Q42080	ATB2; oxidoreductase	Response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Catalytic activity; oxidoreductase activity	Cell part; cytoplasmic part
IPI00520641	P25819	Catalase-2	Cell death; cell redox homeostasis; cellular homeostasis; cellular response to hydrogen peroxide; cellular response to oxidative stress; cellular response to reactive oxygen species; cellular response to stress; death	Antioxidant activity; catalase activity; oxidoreductase activity; peroxidase activity	Cell part; chloroplast; cytoplasmic part; cytosolic part; peroxisome; plastid
IPI00546413	Q8LPS0	CR88; ATP binding	Response to abiotic stimulus; response to chemical stimulus; response to chlorate; response to heat; response to inorganic substance; response to light stimulus; response to osmotic stress; response to radiation; response to salt stress; response to stimulus; response to stress; response to temperature stimulus; response to water; response to water deprivation	Adenyl nucleotide binding	Cell part; chloroplast envelope
IPI00534642	Q9S7E4	Formate dehydrogenase, mitochondrial	Response to cadmium ion; response to chemical stimulus; response to inorganic substance; response to metal ion; response to stimulus	Coenzyme binding; formate dehydrogenase	Cell part; chloroplast
IPI00535542	P41917	GTP-binding nuclear protein Ran-2	Cellular response to stimulus; response to abiotic stimulus; response to cadmium ion; response to chemical	GTPase activity	Apoplast; cell junction; cell part

			stimulus; response to inorganic substance; response to metal ion; response to osmotic stress; response to salt stress; response to stimulus; response to stress		
IPI00543293	P22953	Heat shock cognate 70 kDa protein 1	Defense response; defense response to bacterium; defense response to fungus; response to biotic stimulus; response to cadmium ion; response to chemical stimulus; response to cold; response to fungus; response to heat; response to inorganic substance; response to metal ion; response to stimulus; response to stress; response to temperature stimulus; response to virus; stomatal closure; stomatal movement	Adenyl nucleotide binding	Apoplast; cell junction; cell part
IPI00537112	P55737	Heat shock protein 81-2	Cellular response to calcium ion; cellular response to chemical stimulus; cellular response to inorganic substance; cellular response to metal ion; cellular response to stimulus; defense response; defense response to bacterium; defense response to bacterium, incompatible interaction; defense response, incompatible interactionimmune response ; immune system process; innate immune response; response to abiotic stimulus; response to biotic stimulus; response to heat; response to osmotic stress; response to salt stress; response to stimulus; response to stress; response to temperature stimulus; response to water deprivation; stomatal movement	Adenyl nucleotide binding; catalytic activity; hydrolase activity	Apoplast; cell junction; cell part; external encapsulating structure
IPI00529975	P56777	Photosystem II CP47 chlorophyll apoprotein	Cellular macromolecule metabolic process;	Chlorophyll binding	Cell part; chloroplast part

***The multiple function of the same protein is given in the tables**

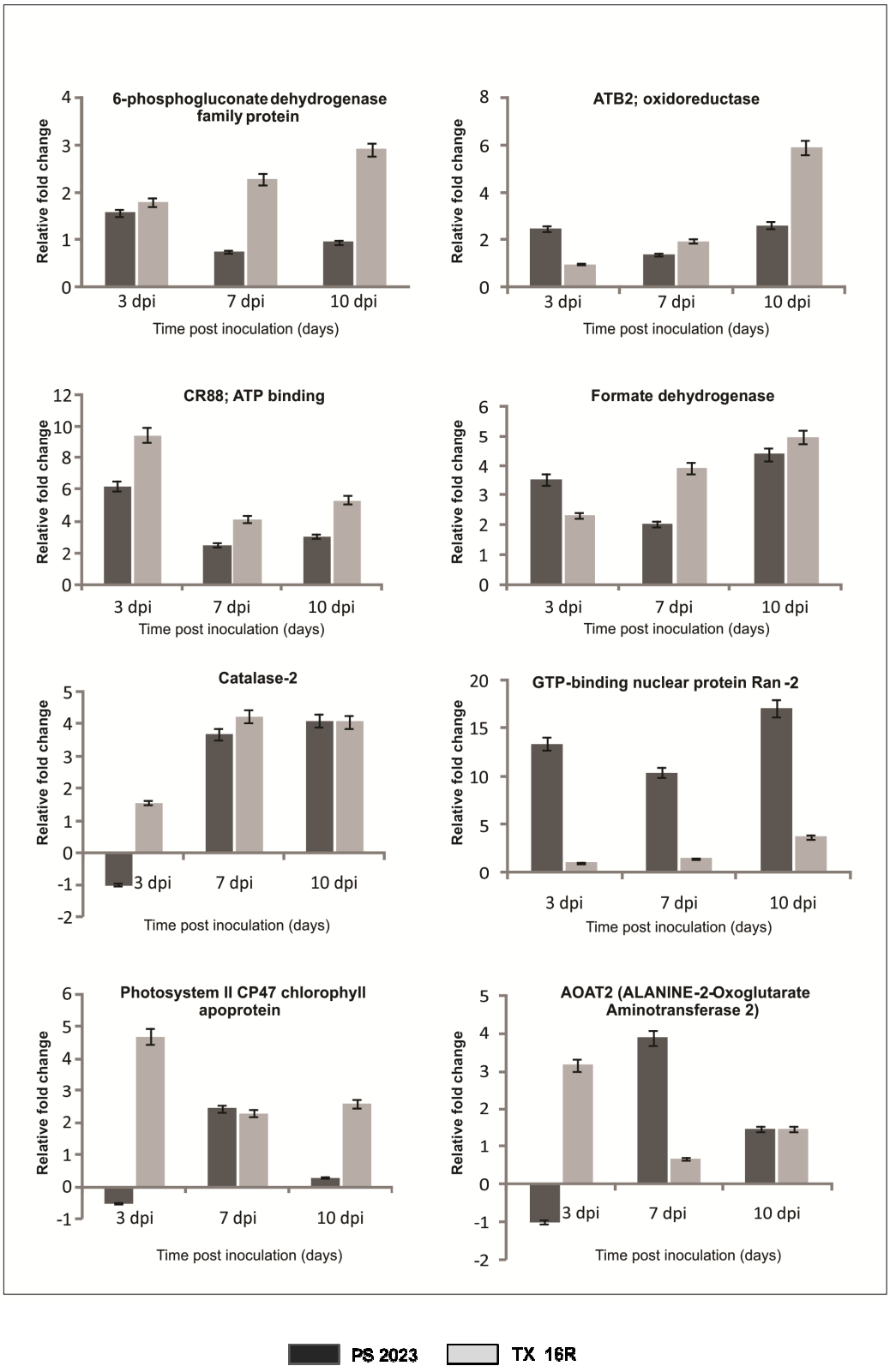

Fig. 6.6 Differential expression of few key stress related proteins in the susceptible (PS 2023) and resistant (TX 16R) genotypes

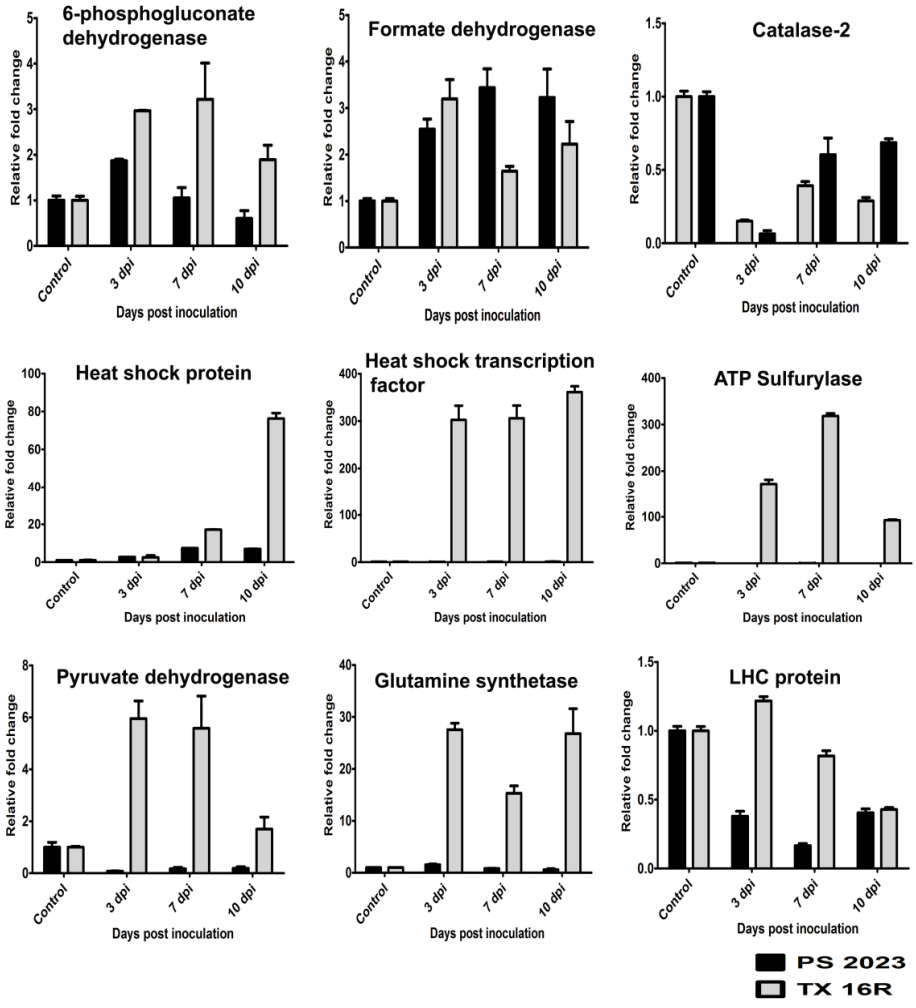

Fig. 6.7 Quantitative real-time PCR (qRT-PCR) validation- Relative expression of genes; 6-phosphogluconate dehydrogenase, Formate dehydrogenase, catalase 2, Heat shock protein, heat shock transcription factor, ATP sulfurylase, Pyruvate dehyrogenase, Glutamine synthetase and LHC protein in Susceptible (PS 2023) and Resistant (TX 16R) in response to powdery mildew infection.

6.2. Discussion

Powdery mildew has become a serious problem on sunflower affecting the crop at post flowering stage in the tropics causing serious yield losses. Reliable sources of resistance were identified in cultivated and wild sunflowers (Saliman et al., 1982; Skoric, 1984; Jan and Chandler, 1985; 1988; Mc Carter, 1993; Rojas-Barros et al., 2004; 2006; Christov, 2008; Dedic et al., 2012; Reddy, 2013). Proteomic analysis of powdery mildew infection on a few economically important crops is published (Curto et al., 2006; Bindschedler et al., 2009; Marsh et al., 2010; Wang et al., 2012; Liu et al., 2013). However, reports on transcriptomic or proteomic analysis of powdery mildew infection on sunflower are not available. Powdery mildew is a biotropic pathogen and the molecular basis of this host-pathogen interaction is not well investigated. In the present study, the proteomes of sunflower following powdery mildew infection were analyzed in three genotypes with differential reaction to the pathogen, *Golovinomyces orontii*.

Mass spectrometry (MS) based proteomics is a widely preferred technique to study the proteome of an organism. Labeling techniques provide data by reducing the technical bias (Russell and Lilley, 2012), while label free methods have the advantage of experimental simplicity and applicability to all types of samples (Sandin et al., 2014) besides providing a higher dynamic range of quantification (Bantscheff et al., 2007; Patel et al., 2009). In addition, label free quantification offers excellent analytical reproducibility, good proteome coverage with less false identification rate. The data produced could be used to perform pathway analysis (Bantscheff et al., 2007; Mann, 2009; Patel et al., 2009; Sjodin et al., 2013). The present study had employed a label free quantification method and a total of 753 proteins were identified with a good number of quantifiable proteins.

Proteins were classified using gene ontology, and the categories of biological function were grouped according to the PANTHER-classification system. Gene ontology of cellular component terms showed that majority of the identified proteins fell into the cell part category (53.7%). It showed that more than half of the identified proteins were localized at the ribosomes, the site of protein synthesis. Most of the proteins identified were classified as metabolic proteins (59.4%). Metabolic proteins are highly abundant soluble proteins and are thus, generally well represented in proteomic studies (Coumans et al., 2009). A major group of proteins that responded to powdery mildew infection were those involved in oxidoreductase (20.4%) protein classes and having the molecular function of catalytic activity (58.9%). These classes of

oxidoreductases correspond to enzymes that are likely to be involved in the apoplastic oxidative burst.

Upon attack by pathogen, host plant activates a network of protein pathways to impart resistance. KEGG pathways in response to powdery mildew infection in susceptible and resistant genotypes varied. The main pathways that differed in resistant (TX 16R) with that of the susceptible (PS 2023) genotype were nitrogen metabolism, photosynthesis and plant-pathogen interaction. An increase in nitrogen metabolism was observed in leaves of wheat (Fu et al., 2016), grapevine (Yao et al., 2012) by powdery mildew and *Piper nigrum* infected with *Phytophthora capsici* (Mahadevan et al., 2016). The photosynthesis protein pathway has been reported to regulate the plant defense responses and induced by abiotic signals such as light, temperature and circadian rhythms (Hua et al., 2013). Similar results were observed in earlier investigations (Yao et al., 2012; Mahadevan et al., 2016; Fu et al., 2016). In the present study, the sunflower-powdery midew interaction pathway related proteins were CR-88 and HSP-80 which are important proteins that upon interaction offer resistance response to powdery mildew. The results indicate that these metabolic pathways play a key role in resistance in sunflower in response to powdery mildew infection

In the present study, comparative proteomics revealed differentially expressed proteins in powdery mildew infected sunflower genotypes having varied levels of resistance *viz.,* PS 2023 (highly susceptible), TX 16R (resistant), and *H. praecox* (immune) to the pathogen. The number of differentially expressed proteins related to oxidative stress following pathogen infection were 10, 5 and 1, respectively. The variation in the number of oxidative stress related proteins in susceptible, tolerant/resistant and immune genotypes depends on the level of infection of the pathogen on the host. With a lesser extent of powdery mildew infection, the elicitation of oxidative stress appeared to be low in the immune host. Microscopic observation of fungal growth in PS 2023 (susceptible) showed complete infection of powdery mildew and leaves were fully covered with haustoria and conidiophores at 10 dpi, whereas in TX 16R (resistant/tolerant), the extent of powdery mildew colonization was less progressive at 10 dpi. In resistant/immune genotype (*H. praecox*), conidial germination was not observed till 3 dpi and there was high reduction in progression of powdery mildew with no visible symptoms of the disease even at 10 dpi. These phenotypic differences in powdery mildew infection levels would have contributed to the different number of oxidative stress proteins expressed in the genotypes studied. The results are in agreement with those on pea (Curto et al., 2006)

and wheat (Wang et al., 2012) infected with powdery mildew where the number of proteins expressed in susceptible genotype was higher than in the resistant genotypes.

Among the differentially identified proteins, the majority of proteins expressed in all the three genotypes belonged to oxidative stress and defense related. In general, fungal infection generates a rapid oxidative burst and plants restrict the propagation of reactive oxygen intermediates (ROIs) in order to localize the death and protect the plant from oxidative stress (Chivasa et al., 2006). 6-phosphogluconate dehydrogenase is a key enzyme involved in the carbohydrate metabolic process (Pentose phosphate pathway) and plays a pivotal role in oxidative stress (Liu et al., 2007; Rojas et al., 2014). The levels of expression of this enzyme were significantly upregulated in the resistant genotype (TX 16R) as compared to the susceptible genotype and there was a gradual increase in levels its expression over time. In general, plants possess effective enzymatic detoxifying systems for protection against reactive oxygen species (ROS) generated from both abiotic and biotic stresses. Enhanced levels of 6-PDH probably play an important role in imparting tolerance of TX 16R to oxidative stress induced by PM. ATB_2 oxidoreductase is an enzyme expressed in response to stress. The expression level of this enzyme was higher in TX 16R as compared to PS 2023. The enhanced levels of oxidoreductase in TX 16R might reduce the damage of redundant ROS as reported in other plants (Vera-Estrella et al., 1994; Mukherjee et al., 2010). CR88 is an adenyl nucleotide binding protein that plays an important role in osmotic stress, and its levels are higher in TX 16R as compared to PS 2023. Catalase is yet another important enzyme with siginificant role in stress response (Bindschedler et al., 2009; 2010; Marsh et al., 2010; Milli et al., 2012). The levels of catalase were more or less equal in both resistant (TX 16R) and susceptible (PS 2023) genotypes, although a slightly enhanced level was observed in the resistant cultivar. It is known that antioxidant enzymes are regularly induced in both resistant and susceptible genotypes in response to pathogen attack (Pitzschke et al., 2006; Zaninotto et al., 2006). It is well established that there is an enhanced production of ROS during pathogen invasion and consequently, the observed increase in antioxidant proteins over the time course of infection is likely to reflect the need for detoxification of the reactive oxygen species.

Earlier studies showed the role of mitochondria in hypersensitive response-associated programmed cell death in plants (Lacomme and Santa Cruz, 1999; Xie and Chen, 2000; Lam et al., 2001). Formate dehydrogenase (FDH) is a mitochondrial membrane protein that oxidizes formate to carbon dioxide in the presence of NAD

(Hourton-Cabassa et al., 1998). Investigations in plants suggest that it is a stress responsive protein (Hourton-Cabassa et al., 1998; Hermana et al., 2002). The up regulation of FDH in TX 16R (resistant) and *H. praecox* (immune) in comparison with the susceptible (PS 2023) genotype suggests the involvement of FDH leading to hypersensitive cell death. It was also shown as a positive regulator of cell death and defense responses to pathogen (Kim et al., 2004; Choi et al., 2012; 2014). It is reported that the induction of FDH by pathogen triggers ROS burst, promotes salicylic acid accumulation and results in expression of pathogen related proteins.

Ran GTPase along with its regulating factors and the Ran-binding proteins play important roles in regulating the nuclear processes through the mitotic phase of cell cycle (Clarke and Zhang, 2008; Merkle, 2011). In case of potato, Rx protein, a class of Ran confers extreme resistance to potato virus X (Bendahmane et al., 1995; 1999). Likewise, Rx protein regulates other NBS-LRR proteins that activate the hypersensitive response (Bendahmane et al., 1999). For the function of Rx protein, it requires heat shock proteins like HSP 90, which are also, required for the activity of other NBS-LRR defense proteins (Peart et al., 2002; Lu et al., 2003; Azevedo et al., 2006). Rx-Ran GAP-2 interaction is a key step in recognition and regulation of extreme resistance in potato against the potato virus X (Tameling and Baulcombe, 2007). However, NBS-LRR protein mediated response was independent of Rx-GAP-2 mediated response. In the present study, the levels of GTP-binding Ran-2 were significantly high in susceptible (PS 2023) genotype when compared with tolerant (TX 16R) and immune (*H. praecox*) genotypes indicating that the susceptibility reactions offered by this genotype contradict the results reported for potato (Tameling and Baulcombe, 2007). Hence, the mechanism of resistance has to be studied in detail. The low abundance of heat shock proteins in the susceptible genotype might be a reason for failure of resistance in PS 2023 when compared with the tolerant genotypes.

Heat shock proteins (HSPs) play an important role in the maintenance of cellular homeostasis under stress conditions. Many HSPs perform a key role as molecular chaperones and function in three dimensional folding of proteins altered by the stress within cell, modulating the regulation of stress conditions. The heat shock protein, HSP 70 plays an important role in plant immunity and defense in some plant-pathogen interactions (Islam et al., 2008; Zhang et al., 2015; Yu et al., 2015; Park et al., 2015). Downregulation of HSP 70 was observed in susceptible *Mentha arvensis* infected with *Alternaria* (Sinha and Chattopadhyay, 2011). In the present investigation, higher levels of

HSPs (HSP 70, HSP 80) expressed in TX 16R (tolerant/resistant) with continuous upregulation compared to the low levels (less than 1.5 fold) of HSPs in PS 2023 (susceptible) might provide the defense in the resistant genotype and preventing the pathogen spread in the host.

Light dependent plant immunity is mediated by photosynthesis and photoreceptor signaling (Karpinsk et al., 2003; Roberts and Paul, 2006; Roden and Ingle, 2009; Hua et al., 2013). Several studies on plant-pathogen interactions showed the profound effect of light on plant immunity (Genoud Wu and Yang, 2010; Liu et al., 2011; Kazan and Manners, 2011; Xie et al., 2011; Manfre et al., 2011; Cerrudo et al., 2012; Gohre et al., 2012). Among the three genotypes studied in the present study, *H. praecox*, a wild species which was immune to PM exhibited 50% of differentially expressed proteins that were related to photosynthetic process and up regulated at 7 dpi (Table 5.3). Similar results were obtained in wheat in response to PM attack (Fu et al., 2016). In the present study, photosynthesis related proteins such as light harvesting complex, photo system I, ATRAB7B, apocytochrome f and cytochrome b559 were detected in high abundance in *H. praecox*. The low abundance of photosynthesis related proteins (TX 16R) or down regulation over the time (PS 2023) indicate that the regulation of light reactions in a particular genotype could be related to its level of resistance to PM.

The characterization of differentially expressed genes in tomato against powdery mildew showed the possible role of oxidative burst and hypersensitive reaction (Li et al., 2006). The study showed that the levels of expression of oxidative burst related genes like ascorbate peroxidase, peroxiredoxin and catalase play an important role in both susceptible and resistant genotypes. In the present investigation, low (catalase) or irregular (ascorbate peroxidase) level in PS 2023 (susceptible) might have resulted in failure of the susceptible genotype to utilize the HR response to arrest the pathogen growth. Another important oxidative stress related gene 6-phosphogluconate dehydrogenase (Stover et al., 2011) was up regulated upon pathogen attack and this protein plays a key role in the maintenance of intracellular redox balances and stress tolerance in plants (Stover et al., 2011; Yang et al., 2015). The higher levels of 6-phosphogluconate dehydrogenase in TX 16R (resistant) might have contributed in maintaining the balance oxidative burst. Over expression of formate dehydrogenase in Arabidopsis exhibited greater resistance to bacterial pathogens (Choi et al., 2016). This is required for basal defense and gene mediated resistance to bacterial pathogens. In the

present study, higher levels of formate dehydrogenase were observed in both resistant (TX 16R) and immune (*H. praecox*) genotypes compared to the susceptible accession. FDH was reported as an important stress responsive protein. Expression levels of heat shock genes play an important role in both susceptibility and resistance process. The expression level of heat shock proteins (HSP-70) was reported to be high in resistant genotypes of barley (Gjetting et al., 2004; Hein et al., 2005; Molitor et al., 2011) and tomato (Byth et al., 2001). The abundant protein expression in TX 16R probably is an important contributor of resistance against powdery mildew.

In the present study, quantitative analysis of proteins expressed in sunflower in response to powdery mildew infection was studied. The protein-protein interaction pathway analysis showed that photosynthesis and plant-pathogen interaction protein pathways are important pathways regulated in sunflower resistance in response to powdery mildew. Important proteins related to protein classes like oxidative stress (catalase, 6-phosphogluconate dehydrogenase), defense (HSP-70, HSP-80 and formate dehydrogenase) and photosynthesis were observed in abundance and changed significantly during powdery mildew infection in resistant and immune genotypes. In the susceptible genotype, a basal defense was initiated, which was similar to the response in resistant genotype, but the timing and expression of appropriate proteins were inadequate leading to failure in restricting the disease progression. The present investigation is the first study that unravels the sunflower-powdery mildew disease interaction, which could be exploited further in breeding programmes aimed at incorporation of resistance to powdery mildew.

Chapter 7

Inheritance and molecular mapping of powdery mildew resistance genes in sunflower (*Helianthus annuus* L.)

The present chapter reports on the inheritance of powdery mildew with appropriate population and to mapping the powdery mildew resistance genes using SSR markers and results and discussion were presented in the following chapter.

7.1. Results

7.1.1. Inheritance of powdery mildew resistance

The levels of resistance or susceptibility to powdery mildew was tested in F_2 and RILs ($F_{5/6}$) of the cross PS 2023 and TX 16R and their parental lines based on visual inspection and scores given based on a 0-9 scoring scale of powdery mildew. Powdery mildew disease score distribution of both F_2 and RILs exhibited a continuous variation (Fig. 7.1). The continuous distribution of disease scores in both populations confirms the polygenic control of the resistance. This observation was statistically proved by Sharpiro-Wilk W test for normal distribution (*P=0.05*) for both F_2 and RILs. These observations led to conclude the of quantitative inheritance of powdery mildew resistance in sunflower in the TX 16R accession.

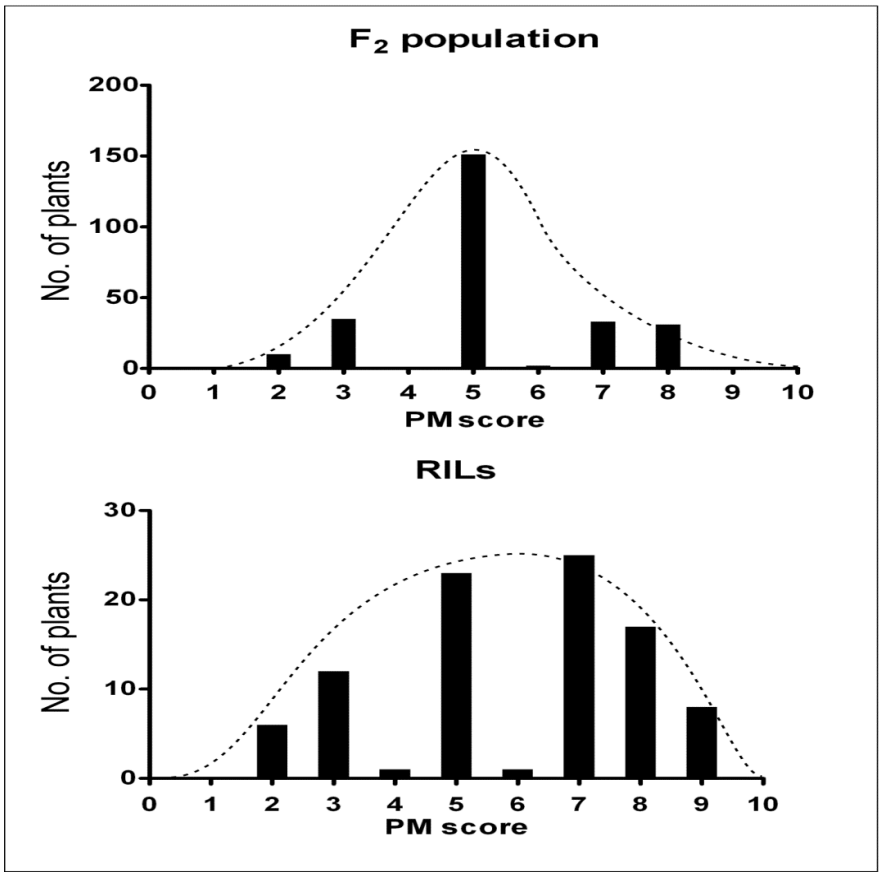

Fig. 7.1 Frequency distribution of disease scores in F_2 and recombinant inbred lines (RILs) derived from a cross between PS 2023 and TX16R

7.1.2. Linkage map construction

The two parents, PS 2023 and TX 16R were screened for molecular polymorphism using 484 SSR markers. A total of 168 primers showed polymorphism among the two parents (Fig. 7.2a). The polymorphism between PS 2023 and TX 16R is 34%. Data obtained from a set of 64 polymorphic SSR markers were selected for Linkage map construction (Fig. 7.2 b, c). The map consisted of 17 linkage groups corresponding to the 17 chromosomes. These 64 markers were evenly distributed on 17 linkage groups (haploid set of chromosomes in sunflower). The constructed maps represented 17 linkage groups spanning 1200 cM.

7.1.3. QTL analysis

The QTL analysis resulted in the identification of three QTLs, distributed in three genomic regions on two chromosomes. Two QTLs were identified on LG-10 with LOD scores of 7.11 and 4.38 (Fig. 7.3) and one QTL on Linkage group 5 (LOD= 3.61) (Fig. 7.4). Thus, these QTLs are involved in the quantitative resistance to powdery mildew (Table 7.3). The three QTLs have a LOD score of 31.80, 57.12 and 23.82% of phenotypic variation in the mapping population for resistance to powdery mildew. All the three QTLs showed positive additive effect (Table 7.3).

Table 7.3. QTLs for resistance to powdery mildew (LOD>3.0)

Trait	Chromosome	Position cM	Left marker	Right marker	LOD	PVE%	Additive
PMR	5	16	ORS 1028	ORS 538	3.61	31.00	1.36
PMR	10	31	ORS 691	ORS 853	7.11	57.12	1.77
PMR	10	70	ORS 853	ORS 78	4.38	23.82	1.08

PMR- Powdery mildew resistance; LOD- Likelihood of odd;

PVE -Phenotypic variation explained

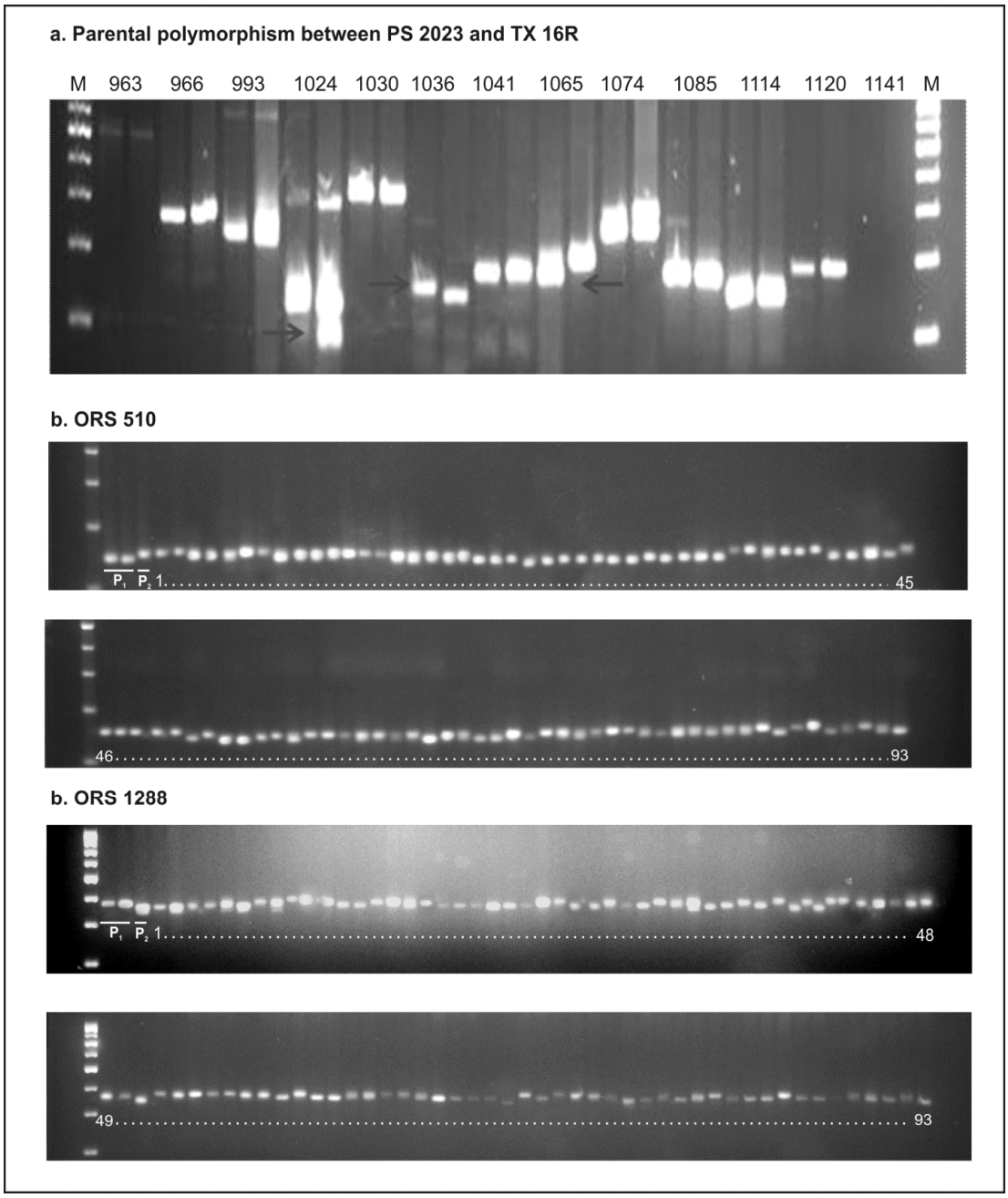

Fig. 7.2 Gel image of the markers showing polymorphism between the parents PS 2023 and TX 16R (a), Gel images of the SSR marker ORS 510 (b) and ORS 1288 (c) markers assayed in the 93 RILs population

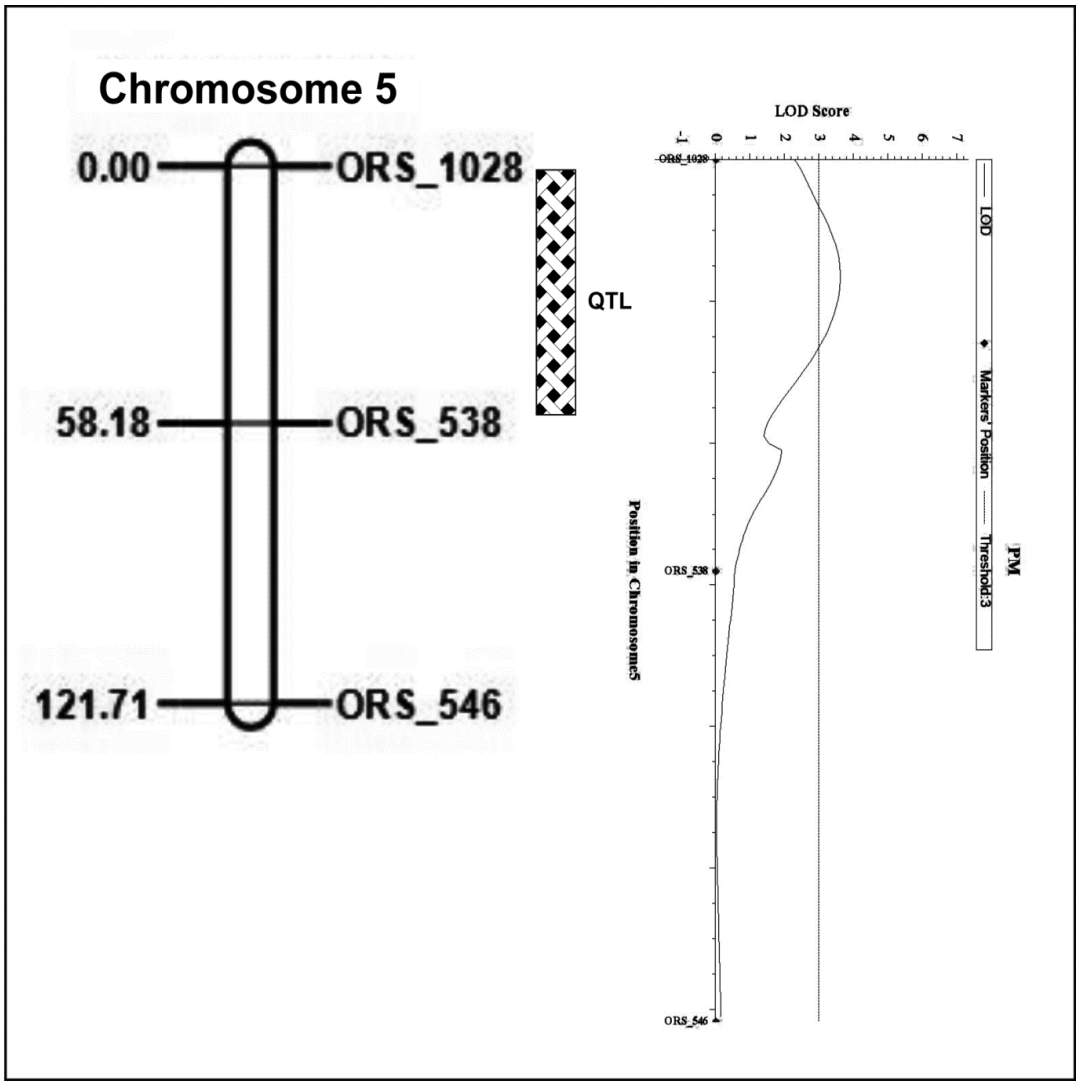

Fig. 7.3 Position of the QTL (LG 5) for powdery mildew resistance in the linkage map derived from RILs population. The distance between the markers is indicated in cM and the LOD significance threshold of 3.0 is also shown.

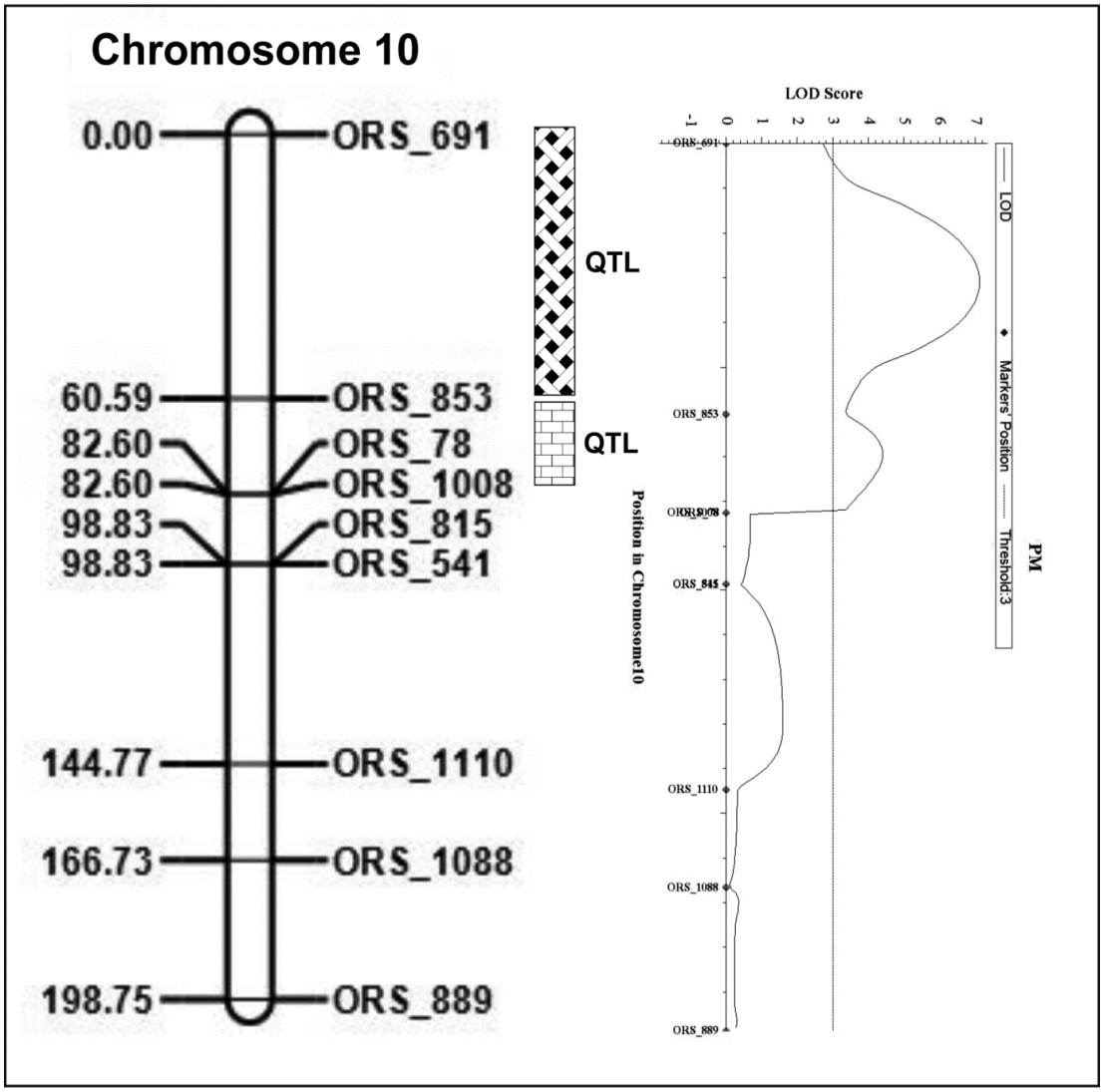

Fig. 7.3 Position of the QTL (LG 10) for powdery mildew resistance in the linkage map derived from RILs population. The distance between the markers is indicated in cM and the LOD significance threshold of 3.0 is also shown.

7.2. Discussion

Powdery mildew has become one of the major problems in sunflower cultivation. Earlier reports are confined to determination of inheritance of resistance to powdery mildew; however, the corresponding resistance genes have not been mapped. One of the main objectives of the present investigation was to study the inheritance of powdery mildew resistance in sunflower and initiative had been taken to map the genes responsible for resistance to powdery mildew by using appropriate mapping population. The inheritance of powdery mildew resistance and putative QTLs identified for resistance to powdery mildew in sunflower are presented.

Information on inheritance of resistance to powdery mildew in sunflower is available and depending on the donor species, mode of inheritance varied. Previous studies had reported partial dominance (Jan and Chandler, 1988), digenic control (Rojas-Barros et al., 2006), single dominant and polygenic inheritance of resistance to powdery mildew (Christov, 2008; Nagayya, 2013). The continuous distribution of disease scores on both F_2 and RILs suggests polygenic control of powdery mildew resistance in the present investigation. The inheritance data of continuous distribution was consistent in both F_2 and RILs. The normal distribution shows that there are many genes for resistance. The low number of resistant lines was due to the cross of a resistant (TX 16R) line with the highly susceptible line PS 2023. In general, a cross between a resistant and highly susceptible parent is expected to result in one resistant to seven susceptible and thus, relatively a large population falls in the susceptible categories.

Several studies reported the mapping genes for resistance to downy mildew, rust and sclerotinia rot in sunflower (Table 7.2). However, there are no reports on mapping genes for resistance to powdery mildew in sunflower. Powdery mildew resistance QTLs are identified in a number of crops, i.e. apple, barley, cucumber, peach, flax, grapes, melon, mungbean, oat, pea, pepper, roses, ryegrass, tomato, watermelon and wheat (Table 7.1). These studies had identified both qualitative and quantitative resistance in these crops. It suggests the existence of multiple mechanisms of resistance to powdery mildew. In sunflower also, both qualitative and quantitative inheritance resistance is reported (Jan and Chandler, 1988; Rojas-Barros et al., 2006; Christov, 2008; Nagayya, 2013). The present study resulted in identification of three QTLs in three genomic regions of two chromosomes *viz.,* LG 5 and LG 10. In addition to LG 5, linkage 10 is of particular interest as this is the linkage group which is reported to be associated with resistance loci for downy mildew and Sclerotinia rot. Vear et al. (2008) had reported one

downy mildew resistance QTL on LG 10. The simple sequence repeat marker ORS 853 was found to be the flanking marker for both the QTLs present in the Linkage group 10, and this region indicates that it is an important regions, which needs further investigations. SSR marker 1008 of LG 10 was closely linked to downy mildew locus PI_{13} (Mulpuri et al., 2009). Both powdery mildew and downy mildew are biotropic fungi that attack sunflower. Also, QTLs for *Sclerotinia* rot resistance QTLs were detected on the LG 10 (Gentzbittel et al., 1998; Bert et al., 2001; Micic et al., 2005). All the three QTLs indentified for powdery mildew resistance were additive. These QTLs combine explained most of the total phenotypic variation with only additive effect.

Since, quantitative resistance is durable (Johnsen 1983; Lindhout 2002), it would be great importance in powdery mildew resistance breeding program in sunflower. In the present study, the genomic regions in the chromosome responsible for resistance to powdery mildew in sunflower was identified. However, for fine mapping of the gene, a large number of markers, population and multilocation screening of the RILs. Identification of molecular markers close to QTLs controlling powdery mildew resistance would provide marker-assisted selection in breeding programs aiming at introgression of resistance from TX 16R to elite breeding lines.. The present study results might be a step ahed in planning a limited genetic strategy (Riaz et al., 2011) to sunflower powdery mildew and its use in marker assisted breeding.

Chapter 8

Conclusions

Screening of about 420 accessions comprising of wild *Helianthus* species, interspecific derivatives, core germplasm, inbred lines and few exotic accessions was done under natural field conditions for two years and further confirmed by screening under artificial inoculation conditions. PCR analysis using primers specific to powdery mildew causing genera gave a 391 bp band which confirmed the pathogen as *G. orontii.* Seven different screening methods were tested which induced infection, but dusting of spores on to the healthy leaves proved to be convenient and a more effective method of infection. Based on the differential response of the accessions derived from diverse genetic backgrounds, a scale for obtaining reliable estimates of the disease has been devised. Among different cultivar germplasm accessions, the disease severity index (DSI) ranged from 15 to 100 and area under disease progression curve (AUDPC) ranged from 95 to 648. Among the four groups of cultivated sunflower accessions tested, DSI and AUDPC was in the order of exotic lines < interspecific derivatives < inbred lines < core germplasm. Reliable sources of resistance to the pathogen were identified in 4 annual wild species (*H. argophyllus, H. agrestis, H. debilis, H. praecox*), 6 perennials (*H. angustifolius, H. atrorubens, H. rigidus, H. salicifolius, H. pauciflorus* and *H. resinosus*), two interspecific derivatives (HIR-1734-2, ID-25) and two exotic lines (TX 16R, EC-537925/USDA-25).

Comparative study of resistant (TX 16R and ID-25) and susceptible genotypes (PS 2023 and Morden) of sunflower to unravel the responses to powdery mildew caused by *Golovinomyces orontii*, plants were subjected to infection with the pathogen and the infection process of *G. orontii* and role of antioxidant enzymes in conferring resistance in the resistant genotype against the pathogen were studied. The extent of powdery mildew colonization on both the susceptible and resistance genotypes was assessed. The relative changes in the reactive oxygen intermediates and the levels of scavenging antioxidant enzymes were quantified. The colony growth was apparently similar in both resistant and susceptible genotypes up to 3 days post infection. At the later time points (4-10 dpi), the growth rate and number of conidiophores per colony in susceptible genotypes increased rapidly compared to the resistant genotypes. The restriction of powdery mildew growth in resistant genotypes suggests that a specific resistance mechanism is operating in these genotypes. The levels of reactive oxygen intermediates like superoxide (O_2^-) and hydrogen peroxide (H_2O_2) were more pronounced in the susceptible genotypes compared to the resistant genotypes indicating a high oxidative burst in susceptible genotypes. This study indicated differential accumulation of antioxidant enzymes in susceptible and resistant genotypes following *G. orontii* infection. The levels of

antioxidant scavenging enzymes like superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) were significantly higher in the resistant genotypes. The synergy between reactive oxygen intermediates and the antioxidant enzymes in resistant genotypes modulates steady state levels of superoxide radicals and hydrogen peroxide thereby protecting resistant plants from oxidative burst and regulating the plant defense against powdery mildew infection.

Proteomic approaches to unravel the plant-pathogen interactions have contributed significantly in understanding the mechanism of resistance. The present study aimed at quantitative proteomic analysis of susceptible (PS 2023), resistant (TX 16R) and immune (*H. praecox*) genotypes of sunflower in response to powdery mildew infection at 3, 7, 10 days post infection. The majority of differentially expressed proteins in the resistant genotype belonged to oxidative stress, defense and photosynthesis. In case of immune genotype, 50% of proteins were related to photosynthesis, which play a key role in plant immunity. A few similar proteins were also expressed in the susceptible genotype, but in their reduced abundance besides being inadequate in timing of expression probably leading to its susceptibility to powdery mildew. KEGG enrichment analysis showed that carbon metabolism, photosynthesis and plant pathogen protein pathways are the key pathways in governing the resistance. The present study has provided information on the key proteins that are upregulated in resistant and immune genotypes, which restrict the disease progression and contributes the first quantitative proteomic data of sunflower-PM infection process.

Despite its yield losses in the crop, a few studies have reported on the identification of sources of powdery mildew resistance and their inheritance. The genes conferring reistance to their pathogen were not mapped. The objectives of the present investigation also included a study on the inheritance of powdery mildew resistance and mapping the quantitative trait loci (QTLs) governing resistance to powdery mildew (*G. orontii*). The inheritance was observed as a continuous distribution by using 264 F_2 population and 93 F_6 recombinant inbred lines (RILs) of a cross between highly susceptible accession PS 2023 and the resistant accession TX 16R. The linkage map was constructed with 93 RILs. The map spans 1200 cM and consists of 64 markers distributed along the 17 sunflower chromosomes in the haploid set. Quantitative trait loci analysis identified three genomic regions for resistance to powdery mildew. Among the three QTLs, two were mapped on the chromosome 10 and one was on the chromosome 5. This constitutes the first report on QTL mapping of powdery mildew

resistance in sunflower. It would be very useful in fine mapping and for introgression of resistance in sunflower for powdery mildew through marker assisted breeding.

Finally it has been confirmed that *G. orontii* is the causative pathogen of powdery mildew in India. Germplasm from diverse sources was evaluated for powdery mildew resistance. Resistance to *G. orontii* in resistant genotypes is conferred at later stages in the infection. In both susceptible and resistant genotypes, *G. orontii* spores germinated and developed hyphae. Asexual reproduction was significantly reduced on resistant genotypes in terms of number of conidiophores as well as number of conidia. This indicates that resistance in resistant genotypes affects the fungal growth and spread primarily after the formation of conidiophores and reduced build up of spore load. Important proteins related to protein classes like oxidative stress defense and photosynthesis were observed in abundance and changed significantly during powdery mildew infection in resistant and immune genotypes. The inheritance of powdery mildew resistance in sunflower was observed as polygenic and three major QTLs were mapped on chromosome 5 and 10. Being sources of powdery mildew resistance, these resistant accessions serve as promising donors in breeding for powdery mildew resistance in sunflower.

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Development of a scoring scale for powdery mildew (*Golovinomyces cichoracearum* (DC.) V.P. Heluta) disease and identification of resistance sources in cultivated and wild sunflowers

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Abstract Powdery mildew incited by *Golovinomyces cichoracearum* has become a serious problem on sunflower in India during the past 2–3 years. Genetic resistance in the released cultivars and the parental lines of hybrids is rather limited. Hence, screening of about 420 accessions comprising of wild *Helianthus* species, interspecific derivatives, core germplasm, inbred lines and few exotic accessions was done under natural field conditions for 2 years and further confirmed by screening under artificial inoculation conditions. PCR analysis using primers specific to powdery mildew causing genera gave a 391 bp band which confirmed the pathogen as *G. cichoracearum.* Seven different screening methods were tested which induced infection, but dusting of spores on to the healthy leaves proved to be convenient and more effective method of infection. Based on the differential response of the accessions derived from diverse genetic backgrounds, a scale for obtaining reliable estimates of the disease has been devised. Among different cultivar germplasm accessions, the disease severity index (DSI) ranged from 15 to 100 and area under disease progression curve (AUDPC) ranged from 95 to 648. Among the four groups of cultivated sunflower accessions tested, DSI and AUDPC was in the order of exotic lines < interspecific derivatives < inbred lines < core germplasm. Reliable sources of resistance to the pathogen were identified in four annual wild species (*H. argophyllus, H. agrestis, H. debilis, H. praecox*), six perennials (*H. angustifolius, H. atrorubens, H. rigidus, H. salicifolius, H. pauciflorus* and *H. resinosus*), two interspecific derivatives (HIR-1734-2, RES-834-3) and two exotic lines (PI 642072, EC-537925).

Keywords Core germplasm · Inbred lines · Interspecific derivatives · *Helianthus* species · Powdery mildew · Screening

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops of India. Until recently, the major diseases limiting sunflower cultivation in India were *Alternaria helianthi*, downy mildew, sunflower necrosis disease and occasionally rust. During the past 2–3 years, powdery mildew caused by *Golovinomyces cichoracearum* (DC.) V.P. Heluta (formerly *Erysiphe cichoracearum*) has become a serious problem on sunflower in the major sunflower growing regions in Southern India. In India, the occurrence of powdery mildew was first reported on Mexican sunflower in the year 2008 (Baiswar et al. 2008) and subsequently on cultivated sunflower in 2009. In the first 2 years of its

occurrence, it was observed only during spring season and at flowering and post flowering stages, but subsequently it has become serious during all crop growing seasons and often infecting the crop at vegetative stage itself.

Powdery mildew is worldwide in its distribution, but the greatest severity has been observed in tropical parts of the world, where it advances senescence of the plant at the flowering or post flowering stages (Zimmer and Hoes 1978; Gulya et al. 1997). Powdery mildew was the second most established disease in 1989 in California and was frequently observed every year between 1983 and 1989 (Gulya et al. 1991). The favorable condition for powdery mildew occurrence in Mexico resulted in significant losses in seed yield up to 13 % (Diaz-Franco 1980). A field survey on prevalence of powdery mildew at farmer's field in seven districts of Karnataka state, India which is the largest sunflower growing region recorded 30–74 % disease severity (Dinesh et al. 2010). Since powdery mildew in its extreme severity is observed mostly at post anthesis stage, disease control measures are generally not followed. Since the disease is spreading in the tropical and sub tropical regions, use of resistant parental lines and hybrids is warranted. Most of the cultivar germplasm accessions available in India are susceptible to the pathogen.

In earlier studies, sources of resistance have been identified in wild sunflowers. In studies of Saliman et al. (1982), the wild species *H. atrorubens, H. californicus, H. ciliaris, H. debilis, H. decapetalus, H. laciniatus, H. laevigatus, H. microcephalus, H. resinosus, H. rigidus, H. simulans* and *H. smithii* showed resistance at field, the two annuals *H. bolanderi* and *H. praecox* ssp. *praecox* showed resistance under artificial inoculation assays while two annual species *H. debilis* Nutt. ssp. *silvestris, H. divaricatus* and the perennial species *H. strumosus* showed resistance under both field and artificial inoculation conditions. Interspecific hybrids between *H. giganteus, H. hirsutus, H. divaricatus, H. salicifolius* and cultivated sunflower showed no symptoms of powdery mildew (Skoric 1984). In the annual wild species *H. debilis*, the resistance was governed by a partial dominant gene and the interspecific hybrid (PM1) between *H. debilis* and *H. annuus* was registered as a source of resistance to powdery mildew (Jan and Chandler 1985, 1988). Among the 36 genotypes of Jerusalem artichoke (*H. tuberosus*), two genotypes showed resistance to powdery mildew (McCarter 1993). Three sub species of *H. debilis* showed resistance to powdery mildew (Rojas-Barros et al. 2004) and these sources were different from that identified by Jan and Chandler (1985). Transfer of powdery mildew resistance genes from *H. debilis* ssp. *debilis* and *H. argophyllus* into cultivated sunflower indicated that resistance in both cases is controlled by at least two genes (Rojas-Barros et al. 2005). Five perennial wild species, *H. decapetalus, H. laevigatus, H. glaucophyllus*, *H. ciliaris, H. tuberosus* and one annual species *H. debilis* showed resistance to powdery mildew; resistance in *H. decapetalus* was controlled by a dominant gene where as the resistance in the other species was quantitative (Christov 2008). Resistance to this pathogen was also found in some accessions of *H. tuberosus, H. praecox* and *H. bolanderi* (Acimovic 1998). Screening of a total of 333 accessions of 23 perennial *Helianthus* species against powdery mildew under natural infection conditions showed resistance in 124 accessions representing 23 perennial species (Dedic et al. 2012). The studies till date report the sources of resistance from wild species while reports on resistance sources in cultivated germplasm are lacking. However, it is interesting to note that some of the diploid annuals that are easily crossable with cultivated sunflower (*H. debilis, H. praecox* and *H. bolanderi*) confer resistance to the pathogen (Saliman et al. 1982; Jan and Chandler 1985, 1988; Rojas-Barros et al. 2004, 2005; Christov 2008).

Powdery mildew is cosmopolitan in nature. The order Erysiphales contains one family, the Erysiphaceae and 19 genera (Kirk et al. 2008). Identification of powdery mildews on members of the Asteraceae is not simple. It is reported that at least three genera of powdery mildews *viz., G. cichoracearum, Leveillula taurica* and *Podosphaera xanthii* are the causative agents of powdery mildew in sunflower (Saliman et al. 1982; Yang et al. 1988; Gulya et al. 1991; Fang 1973; Anonymous 1994; Braun 1995). Before embarking on a large-scale screening program it is necessary to confirm the pathogen causing the disease. The molecular detection methods for identification of the pathogens based on ITS sequences were found to be superior over microscopic methods (Hirata and Takamatsu 1996; Takamatsu and Kano 2001; Chen et al. 2008). The set of ITS primers designed by Chen et al. (2008) were used in this study to confirm the genus that caused epidemics of powdery mildew on sunflower in Southern India.

The present study was undertaken with the objectives of confirmation of the genus causing powdery

mildew, development of a scale for disease scoring, determination of reliable method of screening; and identification of sources of resistance in wild and cultivar germplasm under natural conditions followed by their confirmation through artificial assays.

Materials and methods

Plant material

The plant material used in the study included 59 accessions of wild *Helianthus* species being maintained at the Directorate of Oilseeds Research, Hyderabad, India, cultivar germplasm lines that included prebred lines, a core subset, exotic lines and interspecific derivatives. Prebred lines consisted of 195 lines developed from annual species at the Directorate of Oilseeds Research. The core subset of cultivated sunflower was obtained from USDA-ARS, Ames, USA and consists of 112 accessions, representing 38 countries of origin. The core subset included two ornamental accessions, seven breeding lines, 12 land races and 91 cultivars (Seiler and Jan 2010). This subset represents wide variability for several qualitative and quantitative traits. Nineteen exotic lines with differential reaction for biotic stresses like rust and downy mildew were also included. Additionally 35 interspecific derivatives developed at USDA-ARS, Fargo, USA and derived from 12 different wild species, including perennial species and different ploidy groups were screened.

Confirmation of the pathogen

For microscopic observations of the pathogen, infected leaves were scraped gently to dislodge the conidia along with the hyphae. The conidia were stained with lactophenol cotton blue and observed under light microscope (Leitz, Diaplan). The procedures for DNA extraction, primers used and PCR amplification were as described by Chen et al. (2008) with minor modifications. Total genomic DNA was extracted from diseased leaf and mycelia scraped from the infected leaves of the susceptible cultivar, PS 2023 following the standard CTAB method with minor modifications (Doyle and Doyle 1990). The material was ground in liquid nitrogen, then homogenized in 20 ml of extraction buffer (2 % CTAB, 20 mM EDTA, 2 % PVP, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0 and 1 % β-mercaptoethanol) and incubated at 65 °C for 1 h. The supernatant was twice extracted with chloroform:isoamyl alcohol (24:1 v/v) and treated with RNase A (100 µg/ml), incubated at 37 °C for 30 min. The DNA was precipitated with isopropanol and washed twice with 70 % ethanol. The pelleted DNA was air dried and resuspended in 500 µl of sterile MilliQ water and stored at −20 °C. The DNA concentration was determined electrophoretically using known amount of λ DNA as standard. The internal transcribed spacer (ITS) of nuclear ribosomal DNA regions was amplified from powdery mildew of sunflower using the powdery-mildew-specific ITS universal primer pair PN23 (5′-CAC CGC CCG TCG CTA CTA CCG-3′)/PN34 (5′-TTG CCG CTT CAC TCG CCG TT-3′) and pair of primers, S1 (5′-GGA TCA TTA CTG AGC GCG AGG CCC CG-3′)/S2 (5′-CGC CGC CCT GGC GCG AGA TAC A-3′), G1 (5′-TCC GTA GGT GAA CCT GCG GAA GGA T-3′)/G2 (5′-CAA CAC CAA ACC ACA CAC ACG GCG-3′), and L1 (5′-CCC TCC CAC CCG TGT CGA CTC GTC TC-3′)/L2 (5′-CTG CGT TTA AGA GCC GCC GCG CCG AA-3′), that were specific to the ITS regions of *P. xanthii*, *G. cichoracearum*, and *L. taurica*, respectively. The PCR reaction mixture contained 0.15 mM dNTPs, 0.4 µM primers, 1 U *Taq* DNA Polymerase, 1× PCR buffer with 1.5 mM $MgCl_2$ (Bangalore Genei, India), and 10 ng of template DNA and sterile distilled water to a final volume of 25 µl. PCR amplification was carried out in a thermal cycler (Applied Biosystems GeneAmp 9700) using the following amplification conditions: 5 min at 94 °C for the initial denaturation, followed by 30 cycles consisting of 40 s of denaturation at 94 °C, 1 min of annealing at 62 °C, and 1.5 min of extension at 72 °C with a final extension at 72 °C for 5 min. The PCR amplified products were resolved by electrophoresis on 2.0 % agarose (Bangalore Genei, India) gel in 1× TAE buffer by electrophoresis at 100 V for 3 h and visualized with ethidium bromide staining. The gel images were recorded using the Alpha Innotech Fluorchem gel documentation system.

Field evaluation

Screening of *Helianthus* species and cultivar germplasm for reaction to powdery mildew was carried out under natural disease incidence conditions at the research farm of the Directorate of Oilseeds Research,

Hyderabad, India during the months of October to March in the years 2010 and 2011 with Morden (early maturing open-pollinated variety), DRSF-108 (population) and the interspecific derivative PS 2023 (derived from *H. petiolaris*) as the susceptible checks. These months had the most favorable condition for powdery mildew infection with a mean temperature of 24 °C ranging from 16 to 33 °C. The relative humidity ranged from 35 to 74 %. The mean sunshine hours were 8, the mean wind speed was 2.9 km/h with an evaporation rate of 3.2 mm. Each entry was planted in a single row of 5 m length spaced 60 cm apart with a plant-to-plant distance of 20 cm and was replicated twice. At flowering stage when disease symptoms started appearing, the field was encaged with a white net (1 mm pore size) to enhance the spore load and disease intensity. Disease severity was recorded at weekly intervals from flowering till maturity stage. The leaf area and days to 50 % flowering of each line were recorded.

Infection methods for artificial assays

Sources of resistance identified in the cultivated germplasm in the field and all the wild accessions were screened under controlled conditions, following artificial inoculation. In order to select the most effective and convenient method of infection for artificial screening, seven different methods described by Karuna (2010) were tested on a susceptible variety (Morden) and two resistant lines (RES-843-3 and PI 642072 which are monoheaded and branched, respectively). These methods were (i) dusting method where infected leaves with conidia were dusted on test leaves moistened with water; (ii) spraying of spore suspension (10^6 conidia/ml) that was prepared by washing infected leaves with sterile water and filtering through double layered cheese cloth; (iii) swab method in which spore solution (10^6 conidia/ml) was gently applied on healthy test leaves with cotton wool; (iv) dipping leaves of healthy test plants in spore solution (10^6 conidia/ml) for 5 min; (v) stapling method in which infected leaves were stapled on to healthy leaves and covered with polythene bags overnight; (vi) blotter paper method in which blotter paper dipped in the conidial suspension (10^6 conidia/ml) was placed on the test leaves; (vii) gentle rubbing of leaves of the healthy plants against infected leaves with conidia. Care was taken that leaves with pathogen were not too old and were not collected under wet weather conditions as it prevents release of conidia from the conidiophores. Among these methods, dusting of spores on test plant leaves proved to be convenient and effective. Hence, the wild species and germplasm lines that showed resistance under field conditions were further evaluated for resistance under artificial conditions by the spore dusting method. About 6–8 weeks after sowing, plants were infested by dusting conidia from heavily infected leaves. Following inoculation, the plants were covered with polythene bags and the relative humidity (RH) of the green house was maintained at 60–70 % for 48 h with occasional misting at regular intervals. The polythene bags were removed thereafter and a temperature of 26–30 °C and RH of 60–70 % were maintained throughout the experimental period. Since there were differences in the level of infection in the wild *Helianthus* species, spore counts per unit area were made. For this, three discs of infected leaves were punched with the eppendorf tube (1.5 ml) cap and immersed in 500 µl of sterile water in the same tube. The material was vortexed to dislodge the spores and the supernatant was collected. The process was repeated twice and all the three pooled samples were used for spore count. Spore count was determined using standard haemocytometer and represented as spores/cm^2. The perennial species *H. strumosus* (STR-15) was highly susceptible and harboured the pathogen throughout the year and hence, served as a continuous source of fresh inoculum.

Disease assessment

For scoring the powdery mildew incidence, a disease scoring scale (0–9) was developed. With regard to disease infection level, the material was scored as 0 = no infection on leaves; low (L) = if leaves had <10 % infection; moderate (M) = if leaves had 11–30 % infection, and high (H) = if leaves had 31–100 % infection. Powdery mildew severity was scored at 10 days interval from 45 days of sowing till plant maturity.

Statistical analyses

Disease was scored on fifteen plants according to 0–9 scale (0 = immune; 1 = highly resistant, 2 = resistant; 3–4 = moderately resistant; 5–9 = susceptible/highly susceptible). Disease severity index (DSI) was calculated according to Kim et al. (2000) using the following formula; and the final severity (disease intensity at the final score) is presented.

$$\text{DSI} = \frac{\sum \text{Ratings for each plant}}{9 \times \text{Number of plants rated}} \times 100$$

where $\sum$ is the sum of the disease rating of the plants, 9 is the highest disease rating.

The progression of powdery mildew disease over time was determined as the area under disease progression curve (AUDPC) according to the formula of Campbell and Madden (1990) as follows.

$$\text{AUDPC} = \sum_{i=1}^{n} [(x_i + x_{i-1})/2](t_i - t_{i-1})$$

where n is the number of evaluation times, x_i is the disease intensity at the evaluation time and $(t_i - t_{i-1})$ is the duration between each evaluation.

DSI and AUDPC were calculated using Microsoft Excel software 12.0. Data on spore counts were subjected to analysis of variance (ANOVA) by using SAS 9.3 package (SAS Institute Inc. USA). Each replication had observations on 10 plants and each treatment had three replicates. The treatment means were compared using DMRT at $P < 0.001$. Correlations between DSI, AUDPC, days to 50 % flowering and leaf area were determined by using Pearson correlation coefficient at $P < 0.001$.

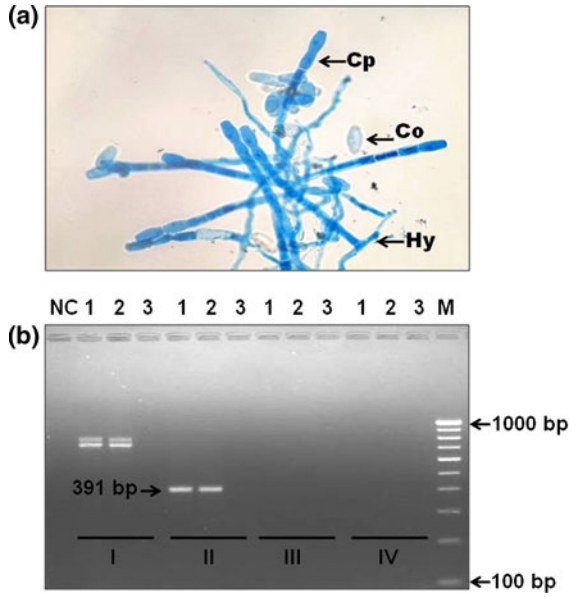

Fig. 1 **a** Light microscopy of *G. cichoracearum* (lactophenol cotton blue stained) with conidiophore (Cp), conidia (Co) and hyphae (Hy). **b** PCR confirmation of *G. cichoracearum* infection using ITS primers (*M* 100 bp marker, *NC* negative control, *1* powdery mildew fungi, *2* sunflower leaf infected with powdery mildew, *3* sunflower leaf free of infection and *I* amplification with ITS primers specific for all powdery mildew fungi, *II* amplification with ITS primers specific to *Golovinomyces cichoracearum*, *III* amplification with ITS primers specific to *Leveillula taurica*, *IV* amplification with ITS primers specific to *Podosphaera xanthii*

Results

Confirmation of the pathogen

The microscopic examination (Fig. 1a) of the fungus showed erected conidiophores with chains of conidia and the conidia were cylindrical. The basal septum of the conidiophores was adjacent to the mycelium and there were no fibrosin bodies, which are typical morphological features of *Golovinomyces cichoracearum*. The universal powdery mildew primer pair failed to give specific bands; and it always gives a non-specific amplicon which cannot be used for detecting specific genera (Chen et al. 2008). A PCR product of 391 bp (Fig. 1b) was amplified with *G. cichoracearum* specific ITS primer pair while there were no PCR amplification products with *Leveillula taurica* and *Podosphaera xanthii* specific ITS primers. Subsequently, pathogen samples obtained from resistant and susceptible cultivars including the wild species and also from other regions in Southern India were also subjected to microscopic and molecular analysis which confirmed that the pathogen causing powdery mildew in sunflower in Southern India is *G. cichoracearum*.

Infection methods for artificial assays

All the seven methods used for artificial infection resulted in infectivity. The spore load (conidia/cm^2) varied from 2,500 to 4,460, 6,000 to 8,600 and 61,100 to 85,990 in RES-843-3, PI 642072 and Morden, respectively (Table 1). Differences in spore load were significant among the genotypes tested and treatment differences within the genotypes with regard to the methods of infection were also significant. Of all the methods, stapling method recorded significantly higher spore load in the three genotypes probably due to overestimate of the spores that dislodge from the stapled infected leaf. However, stapling and blotter paper methods caused mechanical damage and leaf vitrification; respectively. Dipping the leaves in spore

suspension, swabbing with cotton wool, spraying the spore suspension and touching the leaves of test plants with infected leaves resulted in delayed sporulation even in the susceptible lines. Dusting of powdery mildew conidia on healthy leaves resulted in uniform and good levels of infection without any tissue damage. Hence, the method of conidial dusting from infected leaves was employed for artificial screening of resistant material due to convenience in operation and also the level of infection obtained.

Disease assessment

As the material was from diverse genetic backgrounds exhibiting significant variations in qualitative and quantitative characters, infection was observed on the whole plant or confined till the middle leaves or only to the lower leaves. Hence, a scale was developed based on the level of infection on the bottom, middle and top leaves (Table 2).

Table 1 Spore load on resistant and susceptible genotypes in different artificial infection methods

Infection method	RES-834-3 (Resistant) Conidia/ cm^2	PI 642072 (Resistant) Conidia/ cm^2	Morden (Susceptible) Conidia/cm^2
Dusting conidia on test plant	4,067[a]	7,067[b]	67,500[e]
Spraying the spore suspension	3,067[c]	7,000[bc]	65,280[f]
Swabbing with cotton wool	3,233[bc]	6,700[c]	61,100[h]
Dipping the test leaves in spore suspension	2,500[d]	6,000[d]	70,870[d]
Stapling method	4,460[a]	8,600[a]	85,990[a]
Blotter paper method	2,870 [cd]	7,000[bc]	76,400[b]
Rubbing the healthy leaves with infected leaves	2,600[d]	7,000[bc]	62,400[g]

Means in a column followed by same letters are not significantly different according to DMRT at $P < 0.001$

Reaction of *Helianthus* species to *G. cichoracearum*

Table 3 shows the reaction of wild *Helianthus* accessions to *G. cichoracearum* under field and green house conditions. The wild sunflower species showed variability in their reaction to powdery mildew ranging from immune to highly susceptible reaction. Most of the accessions showed the same type of reaction under both field and artificial inoculation conditions. However the accessions, EGG-1629, HIR-03, MOL-1530, GRA-2043 that were immune and OCC-52, NUT-1517, TUB-15, STR-1934, STR-216 (moderately resistant) under field evaluation, were found susceptible under artificial infection. The accessions PAU-S-869, RES-09 and TUB-1705 that were found to be immune under field evaluation showed highly resistant reaction under artificial conditions with very low infection. The wild species, *H. argophyllus*, *H. angustifolius, H. agrestis, H. atrorubens, H. debilis*, *H. rigidus*, *H. salicifolius* and *H. praecox* were found to be immune. The accessions, *H. pauciflorus* and *H. resinosus* were highly resistant, *H. laevigatus*, showed moderate resistance. The accessions *H. bolanderi*, *H. divaricatus*, *H. eggertii*, *H. hirsutus*, *H. microcephalus*, *H. mollis*, *H. occidentalis*, and *H. petiolaris* subsp. *petiolaris* were susceptible under both natural and artificial assay conditions. Intra-accessional variability was found in some species such as, *H. decapetalus*, *H. giganteus*, *H. grosseserratus*, *H. maximilliani*, *H. nuttallii*, *H. strumosus* and *H. tuberosus.* Though the disease index among some of the wild species was similar, differences were observed in the spore load (Table 3). The Duncan's Multiple Range Test showed significant differences in the spore load levels among the species. Correlation between disease index under artificial conditions and spore load was positive and highly significant ($r = +0.90496$).

Reaction of prebred lines

Among 195 prebred lines, 21 accessions showed moderate resistance while the remaining accessions were found to be susceptible to the disease. None of the lines were immune or highly resistant (Table 4). The mean disease index ranged from 22 to 100 with an average of 68. The AUDPC ranged from 144 to 648 with a mean of 445. The days to 50 % flowering ranged from 48 to 76 with a mean of 59. There was a positive Pearson correlation between DSI and AUDPC

Table 2 0–9 scale for scoring powdery mildew incidence

Position of leaf	Bottom	Middle	Top	Scale	Category
Severity of powdery mildew	High	High	High	9	Highly susceptible
	High	High	Medium	8	Highly susceptible
	High	High	Low	7	Susceptible
	High	Medium	Medium	6	Susceptible
	High	Medium	Low	5	Susceptible
	High	Low	Low	3	Moderately resistant
	Medium	Medium	Medium	4	Moderately resistant
	Medium	Medium	Low	4	Moderately resistant
	Medium	Low	Low	2	Resistant
	Low	Low	Low	1	Highly resistant
	0	0	0	0	Immune

0 = no infection, Low = <10 % infection, Medium = 11–30 % infection, High = 31–100 % infection

(r = 0.99) and a negative correlation of DSI and AUDPC with days to 50 % flowering (r = −0.39, r = −0.39, respectively) (Table 6).

Reaction of core germplasm

Out of 112 accessions of the core germplasm, 7 accessions were moderately resistant while all other accessions were found to be susceptible (Table 4). None of the lines of the core germplasm were either immune or highly resistant to powdery mildew. The DSI ranged from 33 to 98 with a mean of 81. The mean AUDPC was recorded as 538 ranging from 144 to 648. The mean days to 50 % flowering was 61 and ranged from 48 to 83 days while the mean leaf area was 2853 ranging from 166 to 11310. Pearson correlation coefficients were positive between DSI and AUDPC (r = 0.46), DSI and LA (r = 0.23), AUDPC and LA (r = 0.01) while there was negative correlation between AUDPC and days to 50 % flowering (r = −0.01) and a weak positive correlation between DSI and days to 50 % flowering (r = 0.07) (Table 6).

Reaction of exotic lines

A set of 19 exotic lines were evaluated for field resistance to *G. cichoracearum*. Two accessions, PI 6402072 and EC-537925 showed resistance (Table 4), five lines showed moderate resistance and the remaining lines were susceptible. The mean DSI ranged from 15 to 100 with a mean of 48 and mean AUDPC ranged from 93 to 648 with a mean of 310. The mean days to 50 % flowering was 64 ranging from 54 to 73. There was a positive Pearson correlation between DSI and AUDPC (r = 0.99) and negative correlation between DSI and days to 50 % flowering (r = −0.36) (Table 6).

Reaction of interspecific derivatives

Of the thirty five interspecific derivatives that were evaluated for field resistance to *G. cichoracearum,* two accessions, HIR-1734-2 and RES-834-3 showed resistance, seven lines showed moderate resistance while the remaining lines were susceptible (Table 5). The mean DSI ranged from 17 to 95 with a mean of 57. The mean AUDPC ranged from 97 to 625 with a mean of 369. The mean days to 50 % flowering was 65 ranging from 57 to 77 days and the leaf area was 2641 ranging from 632 to 8776. There was a positive Pearson correlation between DSI and AUDPC (r = 0.99) and negative correlation between DSI with days to 50 % flowering (r = −0.36) and LA (r = −0.36); AUDPC and LA (r = −0.37), AUDPC and days to 50 % flowering (r = −0.35) (Table 6).

Table 7 provides the data on sporulation of *G. cichoracearum* on the resistant, susceptible and highly susceptible accessions under artificial infection conditions. The sporulation load was significantly low in the resistant plant material compared to the highly susceptible accessions; the highly susceptible accession (PS 2023) had high spore count on all the leaves. The resistant accessions had significantly low spore load even on the lower leaves, which in most cases was much less than the spore load on the top leaves of the susceptible cultivars. The DMRT showed significant differences in spore load from bottom to top leaves of the plant. Regardless of the category, the spore count

Table 3 Reaction of *Helianthus* species under natural and artificial infection conditions

Species	Accession no	PI number	Plant habit	Ploidy	Disease index		Spore count (no of conidia/ cm^2)
					Natural infection (30 plants)	Artificial infection (30 plants)	
H. agrestis Pollard	AGR-1567	PI 468414	A	D	0	0	0^{s}
H. argophyllus	ARG-19	–	A	D	0	0	0^{s}
H. bolanderi	BOL-1023	PI 649867	A	D	9	9	$44{,}533^{cd}$
H. debilis ssp. *debilis* Nutt.	DEB-689	PI 468689	A	D	0	0	0^{s}
	DEB-692	PI 468692			0	0	0^{s}
H. petiolaris ssp. *petiolaris* Nutt.	PET-550	PI 435825	A	D	9	9	$58{,}900^{a}$
H. praecox ssp. *praecox* Engelm. & A. Gray	PRA-1823	PI 494600	A	D	0	0	0^{s}
	PRA-1824	PI 494601			0	0	0^{s}
H. angustifolius	ANG-1241	PI 468419	P	D	0	0	0^{s}
H. atrorubens	ATR-1902	PI 503206	P	D	0	0	0^{s}
	ATR-1581	–			0	0	0^{s}
H. decapetalus L.	DEC-01	–	P	D, T	5	7	$34{,}667^{efg}$
	DEC-1887	–			9	9	$48{,}500^{b}$
	DEC-1922	–			0	0	0^{s}
	DEC-2054	–			0	0	0^{s}
	DEC-970	PI 649970			3	3	$14{,}533^{p}$
H. divaricatus L.	DIV-1881	–	P	D	7	9	$26{,}467^{ijk}$
	DIV-1885	–			7	9	$20{,}233^{mno}$
H. eggertii Small.	EGG-1629	–	P	H	0	5	$23{,}500^{klm}$
H. giganteus L.	GIG-184	PI 547184	P	D	0	1	$4{,}133^{qrs}$
	GIG-2014	–			8	9	$56{,}800^{a}$
	GIG-2015	–			7	7	$22{,}933^{klm}$
	GIG-2017	–			7	9	$40{,}667^{cd}$
	GIG-984	PI 649984			9	9	$38{,}733^{de}$
H. grosseserratus Mar.	GRA-10	–	P	D	7	8	$30{,}767^{ghi}$
	GRA-2043	–			0	9	$41{,}400^{cd}$
	GRA-2212	–			3	3	$3{,}733^{rs}$
	GRA-977	–			9	9	$25{,}367^{jkl}$
H. hirsutus Raf.	HIR-03	–	P	T	0	5	$17{,}033^{op}$
	HIR-1536	–			7	9	$32{,}067^{fgh}$
H. laevigatus T. & G.	LAE-5709	PI 435709	P	T	3	4	$8{,}647^{q}$
H. maximiliani Schr.	MAX-007	PI 650007	P	D	3	3	$6{,}667^{qr}$
	MAX-010	PI 650010			9	9	$23{,}600^{klm}$
	MAX-11	PI 650011			3	3	$6{,}700^{qr}$
	MAX-1631	–			7	9	$31{,}500^{gh}$
	MAX-2010	–			8	8	$29{,}100^{hij}$
	MAX-30	–			9	9	$21{,}166^{lmno}$
	MAX-33001	–			9	9	$22{,}867^{klm}$
H. microcephalus T.&G.	MIC-1872	PI 503231	P	D	9	9	$44{,}267^{c}$
H. mollis Lam.	MOL-1530	–	P	D	0	5	$22{,}233^{klmn}$
	MOL-1629	–			7	7	$30{,}700^{ghi}$
H. nuttallii ssp. *nuttallii* T. & G.	NUT-023	PI 650023	P	D	0	0	0^{s}
	NUT-05	–			5	7	$23{,}267^{klm}$
	NUT-1517	–			3	8	$31{,}800^{gh}$

Table 3 continued

Species	Accession no	PI number	Plant habit	Ploidy	Disease index		Spore count (no of conidia/ cm^2)
					Natural infection (30 plants)	Artificial infection (30 plants)	
H. occidentalis ssp. *occidentalis* Riddell.	OCC-52	–	P	D	3	9	$55,500^{a}$
H. pauciflorus	PAU-S-869	PI 435869	P	H	0	1	$2,900^{rs}$
H. resinosus Small.	RES-09	–	P	H	0	1	$4,100^{qrs}$
H. rigidus	RIG-1696	–	P	H	0	0	0^{s}
H. salicifolius Dietr.	SAL-241	PI 435872	P	D	0	0	0^{s}
H. strumosus L.	STR-15	–	P	D, H	9	9	$36,233^{ef}$
	STR-1934	–			3	9	$37,667^{de}$
	STR-216	PI 547216			3	5	$18,100^{nop}$
	STR-251	PI 503251			1	1	$4,100^{qrs}$
H. tuberosus L.	TUB-05	–	P	H	7	9	$32,133^{fgh}$
	TUB-07	–			7	7	$28,700^{hij}$
	TUB-15	–			3	9	$49,033^{a}$
	TUB-1705	–			0	1	$3,733^{rs}$
	TUB-2069	PI 547243			9	9	$30,933^{gh}$
	TUB-2729	AMES2729			7	7	$32,900^{fgh}$

Ploidy: D = diploid (n = 17), T = tetraploid (n = 34), H = hexaploid (n = 51)

Growth habit: A = annual, P = perennial

Means in a column followed by same letters are not significantly different according to DMRT at $P < 0.001$

was 2–3-fold less in the top leaves as compared to that on the bottom leaves. However in case of RES-834-3 and PI 642072, the disease failed to spread to the top leaves. This observation emphasizes the importance of considering the progression of the disease on the plant in devising a scale for disease scoring. Hence, the present study gives a broad scale (0–9) for disease scoring which allowed identification of resistance sources to *G. cichoracearum* based on the leaf area infected as well as the progression of disease on the plant.

Discussion

Powdery mildew is a frequently found disease on sunflower in warmer regions of the world (Zimmer and Hoes 1978). The disease usually appears at post flowering stage when senescence of lower leaves occur and is reported to cause limited damage in temperate climates. However, the impact of the disease is dramatic in the tropical areas since the powdery mildew instructs the plant to direct energy to the sites of infection (Gulya et al. 1997; http://grdc.com.au). In India, the disease has become serious and occurring regularly since the past 3 years and is observed during different crop growing seasons and under severe conditions is found infecting the cotyledonary leaves as well. Application of fungicides to manage the disease involves high cost, besides the environmental concern and the insensitivity built up in the pathogen limit their usage (Gullino and Kuijpers 1994). Hence, there is a need for identifying reliable sources of resistance to powdery mildew. Wild *Helianthus* species represent a valuable reservoir of genes for several biotic stresses which have been successfully introgressed into cultivated sunflower (Seiler 2008). Sources of resistance to powdery mildew have been identified in both annual and perennial *Helianthus* species but introgression into cultivar background is a long drawn process. Despite the identification of several species as sources of resistance, not much head way has been made in incorporation of resistance for powdery mildew as the disease has not assumed economic importance in the temperate regions till date. The present study has been undertaken for screening and identification of potential sources conferring resistance to powdery mildew in the wild *Helianthus* species and cultivar germplasm by developing reliable screening techniques.

Table 4 Reaction of prebred lines, core germplasm and exotic lines to *G. cichoracearum* under field (net contained) conditions

Plant material	Number of accessions	Disease reaction	Disease index scale	No of accessions	DSI		AUDPC	
					Range	Mean	Range	Mean
Prebred lines[a]	195							
		Immune	0	0	0	0	0	0
		Highly resistant	1	0	0	0	0	0
		Resistant	2	0	0	0	0	0
		Moderately resistant	3–4	21 (PS 1028, 1078, PS 2029, 2037, 2064, 2065, PS 3005, 3008, 3009, 3010, 3011, 3028, PS 4015, 4037, 4050, 4052, PS 5011, 5032, PS 6002, SOC 11, 17)	22–33	27	145–216	175
		Susceptible/highly susceptible	5–9	174	44–100	75	360–648	487
Core germplasm	112							
		Immune	0	0	0	0	0	0
		Highly resistant	1	0	0	0	0	0
		Resistant	2	0	0	0	0	0
		Moderately resistant	3–4	7 (PI 340790, PI 650343, PI 535894, PI 431542, PI 343798, PI 307934, PI 650781)	33–38	35	216–288	236
		Susceptible/highly susceptible	5–9	105	40–98	82	306–648	527
Exotic lines	19							
		Immune	0	0	0	0	0	0
		Highly resistant	1	0	0	0	0	0
		Resistant	2	2 (PI 642072, EC- 537925)	15–22	18	110–144	127
		Moderately resistant	3–4	5 (803-1, EC-687412, EC-53723, EC-53724, EC-53726)	23–27	23	145–155	145
		Susceptible/highly susceptible	5–9	12	44–100	68	334–648	442
Checks		DRSF-108	5–9			86		528
		Morden				93		590
		PS 2023				98		636

DSI disease severity index, *AUDPC* area under disease progression curve

[a] Prebred lines in 1000 series are derived from *H. argophyllus* × cult. sunflower = 61; 2000 series from *H. petiolaris* × cult. sunflower = 32; 3000 series from Wild *H. annuus* × cult. sunflower = 14; 4000 series from *H. argophyllus* × Wild *H. annuus* × cult. sunflower—trispecific = 53; 5000 series from cult. sunflower × *H. debilis* = 17; 6000 series from cult. sunflower × *H. maximiliani* = 6; SOC series from cult. sunflower × *H. occidentalis* = 12; total lines = 195

Table 5 Reaction of interspecific derivatives to *G. cichoracearum* under field (net contained) conditions

DOR Identity	Germplasm	Reg. no.	PI number	Pedigree	DSI	AUDPC
ID-1	ANO-1509-1	GP-135	PI 539894	cmsHA 89*2/*H. anomalus* (Acc. 1509)//RHA 265 F_4	27	185
ID-2	ANO-1509-2	GP-136	PI 539893	cmsHA 89*2/*H. anomalus* (Acc. 1509)//RHA 265 F_4	33	216
ID-3	BOL-774	GP-143	PI 539892	cmsHA 89*2/*H. bolanderi* (Acc. 774) F_3	72	454
ID-4	DEB-CUC-1810	GP-146	PI 539911	nms P21*3/*H. debilis* ssp. *cucumerifolius* (Acc. 1810) F_3	70	447
ID-5	DEB-SIL-367-2	GP-145	PI 539908	cmsHA 89*3/*H. debilis* ssp. *silvestris* (Acc. 367) F_3	52	327
ID-6	DES-1474-1	GP-162	PI 539912	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4	87	576
ID-7	DES-1474-2	GP-163	PI 539913	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4	82	527
ID-8	DES-1474-3	GP-164	PI 539914	cmsHA 89*2/*H. deserticola* (Acc. 1474)//RHA 274 F_4	83	530
ID-9	GIG-1616-1	GP-237	PI 610782	nmsHA P21/*H. giganteus* (Acc. 1616) BC_1F_2	95	626
ID-10	GIG-1616-2	GP-238	PI 610783	nmsHA P21/*H. giganteus* (Acc. 1616) BC_1F_2	83	526
ID-11	HIR-828-1	GP-239	PI 610784	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	51	337
ID-12	HIR-828-2	GP-240	PI 610785	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	30	178
ID-13	HIR-828-3	GP-241	PI 610786	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	82	527
ID-14	HIR-828-4	GP-242	PI 610787	nmsHA P21/*H. hirsutus* (Acc. 828) BC_1F_2	50	317
ID-15	HIR-1734-1	GP-150	PI 539907	cmsHA 89*2/*H. hirsutus* (Acc. 1734) F_3	84	547
ID-16	HIR-1734-2	GP-151	PI 539906	cmsHA 89*2/*H. hirsutus* (Acc. 1734) F_3	21	128
ID-17	HIR-1734-3	GP-152	PI 539905	cmsHA 89*2/*H. hirsutus* (Acc. 1734) F_3	76	464
ID-18	NEG-1255-1	GP-147	PI 539904	nms P21*2/*H. neglectus* (Acc. 1225) F_3	80	527
ID-19	PAR-1084-1	GP-138	PI 539901	cmsHA 89*2/*H. paradoxus* (Acc. 1084) F_4	32	218
ID-20	PAR-1673-1	GP-160	PI 539899	cmsHA 89*2/*H. paradoxus* (Acc. 1673) F_3	32	207
ID-21	PRA-PRA-1142	GP-149	PI 539910	nms P21*2/*H. praecox* ssp.*praecox* (Acc. 1142) F_5	29	211
ID-22	PRA-RUN-417-1	GP-156	PI 539886	cms HA 89*/ *H. praecox* subsp. *runyonii* (Acc. 417) F_3	22	144
ID-23	RES-834-1	GP-153	PI 539897	cms HA 89*2/*H. resinosus* (Acc. 834) F_5	42	272
ID-24	RES-834-2	GP-154	PI 539896	cms HA 89*2/*H. resinosus* (Acc. 834) F_4	42	238
ID-25	RES-834-3	GP-155	PI 539895	cms HA 89*2/*H. resinosus* (Acc. 834) F_5	17	97
ID-26	STR-1622-1	GP-243	PI 610788	nmsHA P21/*H. strumosus* (Acc. 1622) BC_1F_2	85	529
ID-27	STR-1622-2	GP-244	PI 610789	nmsHA P21/*H. strumosus* (Acc. 1622) BC_1F_2	84	563
ID-28	TUB-346	GP-181	PI 564549	cmsHA 89*2/*H. tuberosus* (Acc. 346) F_3	43	238
ID-29	TUB-365	GP-182	PI 564515	cmsHA 89*2/*H. tuberosus* (Acc. 346) F_3	49	285
ID-30	TUB-825-1	GP-245	PI 610790	nmsHA P21/*H. tuberosus* (Acc. 825) BC_1F_2	49	301
ID-31	TUB-825-2	GP-246	PI 610791	nmsHA P21/*H. tuberosus* (Acc. 825) BC_1F_2	78	488
ID-32	TUB-1709-1	GP-183	PI 564517	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_3	81	522
ID-33	TUB-1709-2	GP-184	PI 564518	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_4	70	427
ID-34	TUB-1709-3	GP-185	PI 564519	cmsHA 89*2/*H. tuberosus* (Acc. 1709) F_3	44	237
ID-35	TUB-1789	GP-186	PI 564520	cmsHA 89*2/nms P21 VR1*1/*H. tuberosus* (Acc. 1789) F_3	37	230

DSI = Disease severity index, AUDPC = Area under disease progression curve

Three powdery mildew genera viz., *Golovinomyces cichoracearum (= Erysiphe cichoracearum), Podosphaera xanthii (= Sphaerotheca fuliginea)* and *Leveillula taurica* are identified from diseased sunflower leaves of which *G. cichoracearum* is the most commonly reported in all the continents (Fang 1973; Gulya et al. 1997; Chen et al. 2008). All the three genera are reported from India (Gulya et al. 1997). The symptoms of powdery mildew caused by the three fungi are similar but these fungi can be distinguished from each other based on morphological characters. Chen et al. (2008) has developed a relatively easy and effective technique based on ITS sequence analysis for reliable detection and differentiation of the powdery

Table 6 Correlation coefficients among various characters under field condition on different germplasm sources

Group	Character	Pearson correlation coefficients		
		AUDPC	D/F	LA
Core germplasm	DSI	0.46*	0.07	0.23*
	AUDPC	1.0	−0.01	0.01
Prebred lines	DSI	0.99*	−0.39*	ND
	AUDPC	1.0	−0.39*	ND
Exotic lines	DSI	0.99*	−0.36**	ND
	AUDPC	1.0	−0.37**	ND
Interspecific derivatives	DSI	0.99*	−0.36**	−0.36**
	AUDPC	1.0	−0.37**	−0.35**

DSI disease severity index, *AUDPC* area under disease progression curve, *D/F* days to 50 % flowering, *LA* leaf area, *ND* not documented

* Significance at $P < 0.01$, ** significance at $P < 0.05$

mildew genera. The genus causing the powdery mildew disease on sunflower in India is not known. Based on morphological observations and PCR analysis using ITS primers specific to different powdery mildew genera that infect sunflower, it has been confirmed that *G. cichoracearum* is the causative pathogen of powdery mildew in southern India.

Different disease scoring scales were used for estimation of powdery mildew infection in sunflower viz., a 0–3 scale (Saliman et al. 1982; Dedic et al. 2012), percentage of leaf area infected (Jan and Chandler 1985; Rojas-Barros et al. 2004, 2005; Gulya et al. 1991) and a 0–5 scale (McCarter 1993; Dinesh et al. 2010). In the earlier studies, disease evaluation was confined to wild *Helianthus* species and the material was scored as either resistant or susceptible since only two disease phenotypes were discernible. In the present study, germplasm from diverse sources was evaluated which exhibited variable reaction and based on the observations a visual rating of powdery mildew incidence was assigned on a 0 (immune) to 9 (highly susceptible) scale. This allows researchers to have reliable estimate of the disease based on the percentage of leaf area infected as well as the spread of the disease on the plant. This scale is particularly important in scoring for the disease in mapping populations where the resistance is polygenically controlled. Based on seven different methods tested in the present study, dusting of healthy plants using leaves infected with *G. cichoracearum* was found useful for artificial assays.

Annual *Helianthus* species conferred resistance to diseases like rust and downy mildew while perennial species were found to be resistant to *Sclerotinia sclerotiorum* and *Alternaria helianthi* (Gulya et al. 1997). However in case of powdery mildew, resistance is identified in both annual and perennial species (Saliman et al. 1982; Skoric 1984; Jan and Chandler 1985; McCarter 1993; Rojas-Barros et al. 2004, 2005; Christov 2008; Dedic et al. 2012). The Directorate of Oilseeds Research maintained perennial *Helianthus* species since 1998 in the field garden and none of the species were reported to harbor the disease until 2007. During 2008, there was sudden incidence of powdery mildew in cultivar germplasm and also the wild *Helianthus* species. Although the disease was sporadic and confined to spring season in 2008, it continued to spread to different regions and across the seasons. In the present study based on field

Table 7 Reaction of highly susceptible and highly resistant accessions to *G. cichoracearum* under artificial infection conditions

Accession	DOR identity	Reaction to *G. cichoracearum*	Spore count (conidia/cm^2)			DSI	AUDPC
			Bottom leaves	Middle leaves	Top leaves		
Morden	Morden	Highly susceptible	122,460^{b}	67,692^{b}	53,750^{b}	93	590
PS 2023	PS 2023	Highly susceptible	162,518^{a}	83,653^{a}	72,532^{a}	98	636
PI 331176	SCG-13	Susceptible	93,126^{c}	95,281^{a}	30,917^{c}	46	307
RES-834-3	ID-25	Resistant	36,737^{d}	11,205^{d}	5,106^{e}	17	97
HIR-1734-2	ID-16	Resistant	35,319^{d}	21,844^{cd}	17,872^{d}	21	128
PI 642072	TX16R	Resistant	11,914^{e}	9,503^{d}	3,120^{e}	15	104
EC 537925	USDA-25	Resistant	27,234^{d}	28,652^{c}	1,459^{e}	22	144

DSI disease severity index, *AUDPC* area under disease progression curve

Means in a column followed by same letters are not significantly different according to DMRT at $P < 0.001$

and artificial assays, resistance to powdery mildew was identified in 6 annual and 13 perennial species. Annual species are preferred over perennials due to high crossability success and hybrid fertility. Our study reported high resistance in *H. pauciflorus* (PAU-S-869) for the first time. The accessions DEC-1922, RIG-1696 and SAL-241 were found to be free of powdery mildew both in this study and that of Dedic et al. (2012) but the accessions DEC-1887, DIV-1881, DIV-1885, GIG-2017, GRA-2043, HIR-1536, MAX-33001 and TUB-15 reported as resistant in Dedic et al. (2012) were found susceptible in this study. Results of Dedic et al. (2012) were based on natural infection in the field, while in the present study reaction of wild sunflowers to powdery mildew is based on both natural and artificial infection conditions. Even under natural infection conditions some of the accessions (DEC-1887, DIV-1881, DIV-1885 and HIR-1536) showed contrasting reactions which probably could be due to differences in the virulence of the pathogen or the conditions favouring natural infection in the two test locations which need further investigations. In the present study, intra-accessional variability in the reaction to powdery mildew was found in *H. decapetalus*, *H. giganteus*, *H. grosseserratus*, *H. maximiliani*, *H. nuttallii*, *H. strumosus* and *H. tuberosus* and similar observations were reported by Christov (2008), McCarter (1993) and Dedic et al. (2012). Based on a varied reaction of *H. grosseserratus* and *H. maximiliani* collected from different locations to *Erysiphe cichoracearum*, Saliman et al. (1982) suggests that establishment of different segregated genotypes of these wild species in localized areas might lead to new genetic bases for varied responses to *E. cichoracearum*.

Based on visual rating, the disease score in wild species varied from 0 to 9. In few cases there was no positive correlation between spore count and disease index. For example the species with disease index of 7 had a spore count that ranged from 22,000 to 37,000. Wild *Helianthus* species exhibit wide variability in vegetative and floral characters including leaf characters. Since powdery mildew penetration into the leaf mainly depends on leaf micro morphological characters, differential reaction of the species could be due to variations in the vegetative characters. Chattopadhyay et al. (2011) found highly significant and strong correlation between prevalence of powdery mildew and leaf characters viz., stomatal index, stomatal density and trichome density which were found causal for successful spore penetration of the leaf. As indicated by Fondevilla et al. (2007), diverse resistance offered by *Pisum* species was found to be governed by distinct cellular mechanisms; this probably could be due to several cellular mechanisms involved in plant-pathogen defense mechanisms. Likewise, characterization of defense responses in *Helianthus* species conferring resistance to *G. cichoracearum* is necessary for understanding the mechanism of resistance and broadening the genetic base of cultivated sunflower for powdery mildew resistance.

Among the cultivar germplasm, 11 % were moderately resistant to powdery mildew and none of the accessions studied were found to be immune. Four accessions (PI 642072, EC-537925, HIR-1734-2, RES-834-3) were found to be resistant to *G. cichoracearum.* The accession PI 642072 (Reg no.GP-305) was also registered for its resistance to sunflower rust caused by *Puccinia helianthi* Schw, downy mildew caused by *Plasmopara halstedii* and sunflower mosaic virus (Jan and Gulya 2006). The interspecific derivative RES-834-3 (GP-155) is a derivative of *H. resinosus*. The present study for the first time shows high resistance in *H. resinosus* and it has been reported for its potential tolerance to stem rot, interspecific derivatives HIR-1734-2 (GP-151) was a derivative of *H. hirsutus* and it was already reported for its resistance to *Alternaria helianthi.* Being sources of multiple resistance, these accessions serve as potential donors in resistance breeding programmes in sunflower.

The range of AUPDC values among the germplasm lines ranged from 95 to 648 which indicates the importance of estimating the progress of disease in the germplasm. The positive correlation values between DSI and AUDPC indicates the positive association between the two variables. The negative correlation values between DSI and AUDPC with 50 % days flowering indicates the importance and association of flowering time with progress of the disease (except a weak positive correlation value of $r = 0.07$ between DSI and 50 % flowering of core germplasm). The core germplasm provided a valuable material for screening and understanding the progression of the disease and its association with leaf area and flowering duration. Since, powdery mildew is a foliar disease that occurs at post flowering stages it is important to consider disease progression with these parameters. The correlation values of leaf area with that of DSI and AUDPC varied from core germplasm (positive) and interspecific derivatives (negative) which indicates the

association depending on the material and the extent of variability in the germplasm.

The present study identified new sources of resistance to sunflower powdery mildew in cultivar background which could be utilized in breeding programmes aimed at resistance to powdery mildew. The study resulted in identification of highly susceptible line like PS 2023 derived from interspecific cross involving *H. petiolaris* that could be used as recipient parent in gene tagging and mapping studies as it aids in clear manifestation of the disease. Saliman et al. (1982) replanted the susceptible hybrid sunflower cultivar 894 at 2-week intervals so that ten 2-week-old plants could be inoculated throughout the course of the study. The present study showed the high susceptibility of the perennial wild *Helianthus* species, *H. strumosus* (Acc. STR-15) which could be used as a continuous source of inoculum since powdery mildew fungus being an obligate ascomycetes is difficult to be cultured and maintained under artificial conditions.

For powdery mildew, resistance cannot be relied on a single source as it has been proved that the resistance breaks down rapidly because of the coexistence of sexual and asexual reproduction cycles. Hence, genes responsible for partial resistance are potentially useful for development of cultivars with durable resistance. Other sources including wild species identified in this study are being investigated for their utility in the introgression of resistance to powdery mildew into agronomically desirable backgrounds. Yield losses due to powdery mildew are reported in several crops but have not been assessed systematically for sunflower. Since the disease is spreading in the warmer climates besides making its appearance during all growing seasons and all stages of the crop including cotyledonary leaves, it is important to make yield loss assessments.

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