Chapter-1

Introduction
Plants are required to manage the quality, abundance, balance and diversity of the environment, because in this age of development, a variety of pathogenic microorganisms such as fungi, virus, nematode, bacteria etc. pose a threatening challenge to them. This results in a major decline in the yield of various major crops and also downgrades the quality of their products. Plants are also challenged by a variety of biotic stresses like fungal, bacterial, or viral infections. This leads to a great loss to plant yield. There are various options available for the farmers to protect their crop from the disease. Some options include development of resistant cultivars, biological control, crop rotation, tillage, and chemical pesticides (Thakur and Sohal, 2012). Chemical fungicides have been used by farmers to control fungal diseases, but these fungicides adversely affect those soil microorganisms which are beneficial and also have a negative impact on their ecosystem (Manczinger et al., 2002). Therefore, it becomes crucial to recognize the plant pathology in order to contain plant diseases by means of life cycle control of microorganism, improving pesticide application and breeding for resistance. However, plants also produce defense related secondary metabolites as a method of defense against the pathogens.

Defense mechanism of plants against pathogen is classified into following categories:

1. **Constitutive defense.**
2. **Inducible defense.**

1. **Constitutive defense:** This defense is always present in plants which control infection immediately after exposure of host to pathogen. It protects the plants from invasion of pathogen by the way of performed physical (the cuticle, cell wall, waxy, epidermal cuticle and stomatal aperture) and chemical barrier (including inhibitory compounds or the absence of stimulatory compounds needed for the pathogen development, production of
toxic chemicals and pathogen degrading enzymes). It also provides strength and rigidity to the plant.

2. **Inducible defense:** These defenses are produced when a plant is injured or detect foreign pathogen. It plays an important role in conferring disease resistance against plant pathogen (Maleck and Dietrich, 1999). Inducible defenses are associated with rapid and effective activation of cellular damage response. Activation of induced resistance occurs at the site of initial treatment and in distal, untreated plant parts. Inducible defense can be of two types of resistance i.e.
   a) Localized Acquired Resistance (LAR).
   b) Systemic Acquired Resistance (SAR).

As the name indicates, LAR is mainly expressed locally at the site of infection. SAR is triggered throughout the plant, once the local action has completed. (Thipyapong, 2004). Gene activation in secondary tissue occurs through the plant phloem to the tissue. This triggers onset of an often long lived based spectrum SAR. One of the earliest response of plant to pathogen inoculation is the rapid production of Reactive oxygen species (ROS) which leads to HR and establishment of SAR. Rapid induction of localized host cell death by the HR is one of the major elements of plant disease resistance. Aggregation of high level of salicylic acid during hypersensitive response (HR) leads to the production of defense related proteins, hydrolytic enzymes and other proteins (Wildermuth *et al.*, 2001).

Structural proteins and modification of the cell wall (Ortega *et al.*, 2005) are also included in the HR. The HR helps the plant by sending a signal to the uninfected tissue to activate the SAR (Ryals *et al.*, 2004). Flor (1971) did a novel and passionate work on a plant which is very sensitive to pathogens. As per his observation, the plants have single powerful resistance (R) genes for recognizing particular pathogens containing complimentary avirulence (Avr) genes which encode a protein product. Conditional identification of this protein product, which are encoded by avirulence (Avr) is done by complimentary R gene containing plants only. Thus, the pathogen growth is inhibited by the expression of this defense gene (R). On the other hand, the plant becomes susceptible to the Avr gene containing
pathogen in the absence of this R gene and that pathogen is capable of causing disease (Staskawicz, 2001). Disease resistance mechanism in plants is a complex mechanism constituting many components such as Phytoalexin. Accordingly, on elicitation, induction of many genes encoding phytoalexins, biosynthetic enzymes occur. In plant defense mechanism, a crucial role is played by production of phenolic compounds (low molecular weight, antimicrobial compounds). These phenolic compounds form phytoalexins, which is one of the main classes of secondary metabolites. These compounds are involved in plant protection against stress and contain an aromatic benzene ring with one (phenol) or more (polyphenol) hydroxyl substituent with functional derivatives i.e., esters, methyl esters, glycosides etc. Specific induction of four phytoalexin namely luteolinidin, 5-methoxy luteolinidin, apigeninidin and caffee acids ester of arabinosyl -5-0- apigeninidin, upon inoculation of sorghum seedlings with a non-pathogenic fungus Cochliobolus heterostrophus has been reported by Wharton and Nicholson in 2000. It is well chronicled that these phenolic compounds aggregate as antimicrobial phytoalexins in tobacco virus coat protein inoculated tobacco plants (Ehrenfeld et al., 2005).

Phenolic compounds are the most important group of secondary metabolites involved in both constitutive and induced resistance to pathogen due to their antimicrobial activity. Polyphenol is commonly found in plants. It has been reported to be strong, antioxidant, antibacterial, antimitagenic, antitumor and anticarcinogenic (Kampa et al., 2004). Since phenolic compounds are endogenous, they are of great importance in metabolic activities in plants and also serve as barrier against the microbial invasion (Theerthagiri et al., 2007). Generally, shikimate-phenylpropanoids flavonoids pathways are used for the biogenetic production of these secondary metabolites/phenolic compounds.

As compared to other phenolic compounds, Salicylic acid (SA) plays a significant part in plant defense against infection by pathogen or unfriendly natural conditions (Bosch et al., 2007). Salicylic acid (SA) is an endogenous plant development substance that plays a crucial role in plant growth, advancement and reaction to stresses such as pathogen infection. Chong et al., (2005) noted that salicylic acid is involved in some signal transduction system which induces a
particular enzyme catalysing biosynthetic reaction to form defense compounds such as Polyphenol, alkaloids and pathogenesis-related proteins. Exogenous application of SA has been shown to move systematically through plants, resulting in the expression of a set of defense genes that are activated by pathogen infection and increase in the stress tolerance (Lu et al., 2006). It is reported that SAR mediated signaling pathways play a crucial role in defending the plants against infectious microorganisms such as vital and fungal infection in tobacco, cucumber, Arabidopsis and cotton. Resistance mechanism such as pathogenesis-related (PR) proteins, Phytoalexin production, proteinase inhibitors, or cell wall strengthening and lignification’s is activated by salicylic acid (Sharma et al., 2014), as per the findings of our work on salicylic acid.

Phenylalanine ammonia-Lyase (PAL) \( \text{(E.C.4.3.1.5)} \) is the entry point enzyme in the phenyl propanoid biosynthesis pathway which is also known to modulate the resistance of stress by regulating the biosynthesis of phenolic compounds (Wen et al., 2005) and its presence has been demonstrated in pathogen infected plants (Chen et al., 2000). PAL is the key enzyme in inducing synthesis of salicylic acid (SA) which induces systemic resistance in many plants. The gene was cloned in rice and transgenic rice plants expressing PAL, showed systemic resistance against rice pathogens (Raj et al., 2016). Conversion of L-phenylalanine to various hydroxycinnamic acid is done in three steps through phenylpropanoid metabolism involved in the synthesis of phenolics. Catalysis of the reaction involved in phenylpropanoid pathway is done by phenyl alanine ammonia Lyase (PAL which is specific and precursor enzymes for phenolics biosynthesis. Consequently, this is a significant enzyme responsible to activate the plant defense mechanism. Plants respond to pathogen attack by producing some defense related enzymes. The oxidoreductive property of some enzymes leads to the production of another defense related metabolite that engages in the cell wall polymerization. These enzymes are peroxidases (POx) and polyphenol oxidases (PPO) and some additional enzymes which possess hydrolytic property against pathogen cell wall namely glucanases and chitinases.

Peroxidase (POx) \( \text{(E.C. 1.11.1.7)} \) belongs to Class-9 PR proteins with a wide array of functional and genetic diversity in plants (Scherer et al., 2005). The resistance is associated with the induction of peroxidase in host tissues (Lopez -
Curto et al., 2006). There is growing and strong evidence to show that temporal and spatial control of peroxidase expression is strongly induced by pathogen infection like *Fusarium oxysporum* (Morkunas and Gmerrek, 2007), leading to the establishment of systemic acquired resistance (SAR) (Huang and Backhouse, 2005). The amount of (peroxidase) $\text{H}_2\text{O}_2$ and other enzyme activities increase as per the strength of tolerance or susceptibility to *Fusarium wilt* disease (Subramanian et al., 2006).

Polyphenol Oxidases (catechol oxidase; **E.C. 1.10.3.2**) is an oxidative enzyme. Polyphenol oxidases (PPOs) are copper containing ubiquitous enzymes which utilize molecular oxygen to oxidize common ortho-diphenolic compounds such as catechol to their respective quinones and perform lignification of plant cells during microbial invasion. Based on these browning reactions, PPOs have been suggested to play a defensive role in plants (Lokhandwala et al., 2014). The PPOs may also participate in responding defense reaction and hypersensitivity by inducing plant resistance against fungi (Zheng et al., 2005). They catalyse browning reactions in injured tissues and are of special commercial importance because of their induced browning and quality impairment of fruits, vegetables and fodder plants. The browning reactions occur due to the oxidation of phenolic constituents after damage of cells of intact plant materials. Thipyapong et al., (2004) and Gandia-Herrero et al., (2005) also reported that monophenols are hydrolysed to o-diphenols by these enzymes and also these compounds are oxidized to quinones which are often more toxic to microorganisms than the original phenolic compounds. In addition, polyphenol oxidase and peroxidase are multifunctional enzymes that can prevent biological and chemical attack by raising physical barrier or by counter attacking a pathogen with a high production of free radicals (Passandi et al., 2005).

Biosynthesis and aggregation of different pathogenesis related (PR) proteins primarily indicates towards the presence of a plant defense system. Since the synthesis of PR proteins occur after exposure to plants, they are known to be antimicrobial and are known to consist of around 17 evolutionarily conserved families (Van Loon et al., 2006). Proteins which have basic role in plants can also perform some additional functions in defense. Consequently, the process of cross-linking different parts of the plant cell wall and reinforcing this protective layer in
plants, is performed by extensions and proline-rich proteins (hydroxyproline rich glycoproteins, HyRGOPs) which are called as structural cell wall proteins (Wei and Shirsat, 2006; Deepak et al., 2010). After the pathogen attack (Schenk et al., 2000) and wounding (Cheong et al., 2002) these proteins are positively synthesized but since the cell wall is dynamically composed amid various stages of plant growth, it leads to differences in susceptibility to pathogens.

Oilseed crops are harvested for the producing oil. For example, groundnut, mustard, sunflower, safflower, linseed, soybean and castor are the most significant oil seed crops of India. Amongst all these oil seed crops, Eruca sativa Miller has the most essential medicinal properties and is a drought-tolerant oilseed crop, which initially originated in India and North Africa (Garg G, 2014). E. sativa is originated in Mediterranean region. It is also known as Taramira, Rocket salad, Roquette and white pepper. E. sativa belongs to rapeseed-mustard group and Brassicaceae family (Heta et al., 2017). The crop is grown on soil with reduced fertility and is preferable as compared to other relative species due to its stress nature and adaptability to unfavourable environmental condition (Shinwari et al., 2013).

Classification of Taramira:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
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<tbody>
<tr>
<td>Division</td>
<td>Mangliophyta</td>
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<tr>
<td>Class</td>
<td>Mangliopsida</td>
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<tr>
<td>Order</td>
<td>Brassicales</td>
</tr>
<tr>
<td>Family</td>
<td>Brassicaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Eruca</td>
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<tr>
<td>Species</td>
<td>sativa</td>
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E. sativa is a fast growing, an annual oilseed crop with dull green, deeply cut compound leaves. The leaves are characterized by a distinctive spicy, pungent flavor resembling horseradish. Its tender leaves are used as salad green and its result in stimulant, stomachic and antiscorbutic activity due to their hot pungent taste. Another major use of taramira leaves is in preparation of therapeutic medicines as an
astringent, aphrodisiac, digestive, tonic, emollient, depurative, laxative and rubefacient (Sharma et al., 2012). It is mainly grown in arid and semi-arid region where soil is severely affected and can tolerate temperature less than 4°C. Glucosinolate present in Brassicaceae were found to have anticarcinogenic, antifungal, antibacterial, and antioxidant activities (Garg et al., 2014). The taramira plant also contains high amount of vitamin C, Potassium, Glucosinolate, flavonoids and phenolic compound and is rich in mineral elements. *E. sativa* seed is a rich source of 20-25% protein and 30-35% oil. 96.25% of essential oil extracted from *E. sativa* leaves is 67 volatile compounds (Mitsuo et al., 2002). These volatile compounds contain a high amount of sulphur and nitrogen compounds. Also, *E. sativa* oil contain beneficial enzyme, methylsulphinyl isothiocyanate, which has anticancer activity (Salyogi et al., 2012).

Antioxidative compounds present in *E. sativa*, are believed to possess health promoting properties. In India, it is famous as taramira and is one of the essential edible oilseeds. Skin care industries, cosmetics and soap making industries also make use of taramira oil. It is anti-bacterial, anti-viral, anti-fungal and anti-oxidant, which makes it suitable for medicinal purposes. Since its oil has less cholesterol, it is also used in health food industry. *E. sativa* crops should be grown at optimum temperature of 20°C and germinated at 25°C. August to October is the preferred sowing period for this crop. Due to its high protein content, taramira fixes the amount of atmospheric nitrogen in the soil.

Currently, India and China rank highest globally for the production of *E. sativa*, followed by Myanmar, Sudan, Uganda, Nigeria, Pakistan, Tanzania, Ethiopia and Turkey. India has first position globally, in area and production of *E. sativa*. In year 2010-11 the production of taramira was evaluated about 8.93 lakh tones while in the year 2011-12 it was estimated about 7.31 lakh tones. Taramira is majorly grown in India in the states of Gujarat, Uttar Pradesh, Rajasthan, Madhya Pradesh and West Bengal (Figure1.1).

*E. sativa* is mainly grown in Rajasthan as well as in some parts in Gujarat and Haryana (Sastry, 2003). Major taramira growing district in Rajasthan are Pali, Jalore, Sirohi, Tonk, Kota, Alwar and Ajmer (Figure 1.2). Among other countries, it
is majorly grown in areas spanning from Southern Europe to North Africa, Iran and Pakistan. Total area of taramira crop in India for the year 2013-2014 was 71.30 lakh hectares and Total production of taramira crops in India for the year 2013-14 was 73.00 lakh tones. Average yield for the year 2013-14 was 1023 kg/hectares as against 1007 kg/hectare during the year 2012-13. The largest cultivated area of *E. sativa* crop in India is in Rajasthan. Rajasthan also has the highest productivity of this crop with annual productivity range of 89 kg/ha to 1527 kg/ha from Bikaner to Dholpur district (Anonymous, 2012). Govt. of India announced the minimum support price of taramira for the season as Rs 4500 per quintal.
Figure 1.1: The Major taramira producing states in India
Figure 1.2: The Major taramira producing districts of Rajasthan
The production of *E. sativa* is less than the desired level, but there is potential for a considerably higher yield. Reduced inputs and poor management (e.g. lack of response to fertilization application, irrigation, pest control etc.) could be the cause of low production of this significant crop. Apart from this, other contributing factors towards low production of this crop are the biotic (virus, fungi and bacteria etc.) and abiotic stresses (climatic conditions) and inadequate breeding programs (Pham *et al*., 2010).

Moreover, numerous fungal pathogens pose a great threat to this crop. One of the dreaded disease of taramira, fungal pathogen *Fusarium oxysporum* (a soil born fungus) is the main cause of “Root Rots” disease and it leads to 5 to 60 percent reduction of crop production under the field conditions. Therefore, in order to increase the production of this crop, it is required to protect it from diseases (Dina Karan *et al*., 2005). Plants own defense mechanisms, such as strengthening of the cell wall, ROS production, and programmed cell death, activation of defense genes and synthesis of phenolic or antimicrobial compounds and defense hormones, are also responsible to fight against pathogen attack assault. This makes it necessary to protect the crop against the diseases in order to increase the production. This pathogen’s effect on crop are deadly; leading to plugging of vascular system, yellowing of leaves and ultimately drooping of plants causing huge loss to the economy. In many economically important plant species the biochemical basis of diseases resistance remains unexplored yet. Thus, it is important to understand the biochemical basis of disease resistance of this economically important plant. There are no reports as yet on the biochemical basis of disease resistance against the root rot disease caused by *F. oxysporum* in taramira plants.

**MEMS based sensors for Liquid Chromatography**

Micro Electro Mechanical Systems (MEMS), also known as Micromechanics, Microsystems technology (MTS) or nanotechnology, is an interdisciplinary field of study committed to the physical integration of micromechanical systems with microelectronics, resulting in miniature embedded system that involve micro machined components and structures (Saenz, 2005). MEMS devices, which generally range in size from a couple of micrometres to several millimetres, are made up of
components between 1 to 100 micrometres in size (i.e. 0.001 to 0.1 mm). The world’s smallest guitar is about 10 micrometres long about the size of a single cell.

MEMS devices are fabricated using integrated circuit (IC) batch processing techniques. These systems, which generate effects on the macro scale, can sense, control and actuate on the micro scale level. This technology, which originated in the United States, is known as Microsystems Technology (MST) in Europe and Micro machines in Japan (Partnership Faraday, P, 2002). In the most general form, MEMS consist of mechanical microstructures, micro sensors, micro actuators and microelectronics, all integrated onto the same silicon chip (Figure 1.4).

![Figure 1.4: Schematic illustration of MEMS components (Jain, 2012).](image)

The most popular material used for MEMS is silicon for its semiconductor, physical and commercial properties (Herald, 2015). However, to reduce the cost of the device, polymers, metals, ceramics, glass and plastics have also been explored. The MEMS devices developed to-date can be broadly classified as:

1. Mechanical sensors
2. Physical sensors
3. Chemical sensors
4. Biological sensors BioMEMS
5. Optical MEMS
6. Magnetic sensors
7. RF MEMS
8. MEMS microfluidics
Advantages of MEMS devices
Some of the advantages of MEMS devices are (Herald, 2015):

- Very small size, mass, volume
- Very low power consumption
- Low cost
- Easy to integrate into systems or modify
- Small thermal constant
- Can be highly resistant to vibration, shock and radiation
- Batch fabricated in large arrays
- Improved thermal expansion tolerance
- Parallelism

There are several successful MEMS devices and sensors in the market. Some of them may be cited as: pressure and flow sensors, acoustical sensors, accelerometers, gyros, MEMS mirrors etc. For the current work, we need to have a device capable of carrying out liquid chromatography, and hence covered under MEMS microfluidics. In this category, a CE (Capillary Electrophoresis) tool for the chemical analysis has been developed.

Capillary Electrophoresis (CE)

Capillary electrophoresis is the analysis of charged analytes, inside a capillary, based on their migration under the influence of a direct current electric field. Figure 1.5 is an illustrated diagram of a CE. In this analytical technique, the ions are separated based on their electrophoretic mobility under the influence of an applied DC voltage. Various dependencies of the electrophoretic mobility include the charge of the molecule, the viscosity and the atom's radius. The rate at which the particle moves is directly proportional to the applied electric field, the greater the field strength, the faster the mobility. Magnificent efficiencies and automation are the main advantages of doing electrophoresis in a capillary. The Joule heat dissipation is very efficient due to the small diameters of the capillary which is typically in the inner diameter range of 20-100 μm. This leads to the application of
high voltages up to 3000 V. This leads to fast separations with a very little band broadening. Small bands mean efficient peaks with high plate numbers.

![Capillary electrophoresis](image)

**Figure 1.5:** Capillary electrophoresis (Huan et al., 2012).

Capillary Electrophoresis Chip (CE Chip), a highly-integrated miniature separation and analysis device, comes within the purview of biotechnology and MEMS (Micro-Electro-Mechanic Systems) technology. It has wide applications ranging from disease diagnosis, environment monitoring, new drug development to food safety inspection. There have been varied uses of microfluidics systems in recent years which include various chemical and biological applications, including DNA analysis, capillary electrophoresis, cell cytometry, high throughput screening for combinatorial chemistry, fuel cells, combining multiple biological assays onto a single chip and the generation of multistream segmented flow regimes.

There are five components present in MEMS based CE device:

1. Reservoirs
2. Separation channel
3. Injection channel
4. Electrodes for electrochemical detection
5. Connecting lines and contact pads
1. **Reservoirs** - There are 4 kinds of reservoir - Sample Reservoir, Buffer Reservoir, Waste Reservoir, and Detection Reservoir.

2. **Separation channel** - This separation channel is the place where protein separation takes place. Electrodes are integrated at the second point of the separation channel. Buffer reservoir is attached and assembled on the PC board. Samples during the stationary phase in separation channel give rise to peak profile. Diffusion of the sample from the sample channel is diffused. Buffer stream pushed the sample back into the grounded vial during the stationary phase.

3. **Injection channel** - Injection is the process in which samples are introduced into the capillary for separation of its components. There are two types of injection:
   a. Electro kinetic Injection
   b. Hydrodynamic Injection (Vacuum or pressure injection)

   a. **Electro kinetic Injection** - There are two sides of capillary, one side of capillary is cathode and other side is anode. The sample to be analysed is introduced into the anode of the capillary for applying voltage between anode (containing samples to be analysed) and cathode of the capillary. For analysis, voltage is applied between cathode and anode (containing solution) the EOF starts to move from one tip of the capillary to the other side of the capillary. In addition to the EOF, ions start to move into the capillary for the buffer solution due to electrophoretic mobility. This process is called sample loading. This process is more advantageous when the sample contains small concentration of ions. The duration of this injection for 1-5 sec. In this process, the temperature and viscosity are important parameter for reproducibility. So, it is necessary to control temperature as well as viscosity of the samples to be analysed.

   b. **Hydrodynamic Injection (Vacuum or pressure injection)** - In this process, pressure or vacuum is produced. Pressure is applied at one end with respect to other side end of the capillary. The liquid sample
into the capillary will move due to pressure difference between the two ends of the capillary. Also, temperature and viscosity of sample are important parameter for reproducibility in both injections. So, it is important to control both.

4. **Electrodes for Electrochemical Detection** - Nowadays, electrochemical detection is suited for micro fabrication process and is most common for micro fabrication. Micro electrodes for different shape, size and composition may be patterned on to the glass, silica and any other substrate by photolithography technique to fabricate microchip CE channel. In this way, the electrochemical detection operation can be integrated on microchip. By the use of this technique the CE/ED instruments used are of miniature size, simple electronics principle is required for controlling the data acquisition to perform electro chemical detection. Electro detection can be carried out either by conduction or potiometrically. The CE solution passes over a set of probes and the conductance of the CE solution is measured by the applied voltage.

- **Potentiometric Detection** is for coating the sensing electrode of an ion selective membrane. The potentiometric detection technique measures the potential developed across the membrane or solution interface when the analyte plug passes.

- **Amperometric Detection.** The resulting current generated by either liberation or consumed electrons is measured at the sensing electrode. This technique is for electric potential that has been developed at the reference electrode.

5. **Connecting Lines and Contact Pads:** Driving electrodes along with the metal interconnect were designed to provide necessary electric field for electrophoresis process. These electrodes terminate at the bonding pads placed on the silicon substrate.
The present work is a study on biochemical changes occurring in *Eruca sativa* Miller during root rot disease, after the plant is attached by fungal pathogen *Fusarium oxysporum*. In view of these aspects, the present investigations were conducted with the following specific objectives.

1. Determination of polyphenol and salicylic acid (SA) content in pathogen inoculated and control plants of Taramira (*Eruca sativa*) cultivars of Rajasthan.
2. Qualitative determination of phenolic using TLC and HPLC analysis in the plants as above.
3. Assay of phenylalanine-ammonia Lyase (PAL), peroxidase and polyphenol oxidase enzyme activities in the plant.
4. To study the protein profile of resistant and susceptible cultivars as noted above with special emphasis on PR proteins.
5. Analysis of PR proteins through electrophoresis / MEMS based Capillary Electrophoresis (CE) tool.

The above studies will be conducted under *in vivo* and *in vitro* conditions.