CHAPTER 1

INTRODUCTION

1.1 Pesticide usage

Pesticide residues in agricultural products pose a serious threat to human health all over the world (Zhou *et al*., 2015). According to estimates of the United Nations (UN, 2004), by the year 2075, the total global population may touch the 9 billion mark, which will result in increasing demand for food leading to extensive use of pesticides (Ko *et al*., 2014). *Brassica* spp. are widely attacked by insect pests which results in yield loss. In state of Punjab (India), these yield losses ranged from 6.5 to 26.4 % in the absence of any pesticide (Kular and Kumar, 2011). *Brassica juncea* L., also known as Indian mustard, is an economically important crop which is cultivated as a vegetable and oil yielding crop. During its vegetative growth, it is attacked by various pests *viz.*, soil bugs, termites, turf insects, aphids, leaf hoppers, cut worms etc. The most effective and extensively used insecticide to check these insect pests is imidacloprid (IMI), a neonicotinoid chemical and a systemic insecticide (1-((6-chloropyridin-3-yl)methyl)-4,5-dihydro-N-nitro-1H-imidazol-2-amine) (Ko *et al*., 2014). IMI is colourless crystalline with chemical formula of $C_9H_{10}ClN_5O_2$, molecular weight of 255.7 g mol^{-1} and, its solubility in water is 0.61 g L^{-1} at 20 ^oC. Due to its systemic nature, IMI translocates in plants tissues. Upon ingestion by insect pests, IMI acts on the nervous system of the insects by binding to post-synaptic nicotinic acetylcholine receptors. This results in failure of the nervous system of insects resulting in their death (Gervais *et al*., 2010). IMI is mostly applied to rape, maize and sunflower via soil. Due to its systemic nature IMI helps in controlling both the soil insects and the aerial sucking insects, thus protecting whole plant without creating any aerial pesticide pollution (Heatherington *et al*., 1992; Dewar *et al*., 1996; Bonmatin *et al*., 2005). Recommended field dose of IMI is 20 g active ingredient (a.i.) ha^{-1} for cabbage, eggplant, mustard and rice (Mukherjee and Gopal, 2000; Akoijam and Singh, 2014), 42 g a.i. ha^{-1} for brinjal (Mandal *et al*., 2010) and 80 g a.i. ha^{-1} for grapes (Mohapatra *et al*., 2011). The half-life period of IMI depends upon the nature of soils. It has been reported that half-life of 40 and 124 days in soils without manure and with manure respectively (Rouchaud *et al*., 1994). In

agricultural soils it has been reported to be 69 days, whereas in non-agricultural soils, half-life of IMI ranged from 188-997 days (Gervais *et al*., 2010). Sharma and Singh (2014) reported that after soil application of IMI at the rate of 80 g a.i. ha^{-1}, the IMI residues including its metabolites after 30 and 60 days of treatment were 1.60 and 0.90 mg Kg^{-1} soil respectively. It has been reported that throughout the world, quantity of IMI sold in the year 2008 was ca. 5,450 tonnes (Pollack, 2011). However, global production of IMI in the year 2010 was increased to ca. 20,000 tonnes (Simon-Delso *et al*., 2015). According to latest reports, India exported about 3158 tonnes of IMI from Jan., 2014 to Aug., 2016 (Zauba.com, 2016). In Indian market, IMI is commonly sold with common names like Mida, Comida, Sumida, Imidaplus, Techmida, Filmida, Imidore, Imidon, Glomida etc.

1.2 Pesticide phytotoxicity and its metabolism

IMI gets translocated from soil to the photosynthetic site of the plants via xylem channel and then transported to flowers and fruits through phloem (Laurent and Rathahao, 2003; Alsayeda *et al*., 2008). Pesticides cause toxicity to plants by way of chlorosis, necrosis and vein discolouration, leading to their retarded growth and development by reducing photosynthetic efficiency and, nitrogen and carbon metabolism (Kana *et al*., 2004). Pesticide application was also reported to inhibit photosynthesis by negatively affecting the plant photosystems (Xia *et al*., 2006). Saladin and Clement (2005) reported that pesticides inhibit growth and development of reproductive organs of plants, causing impaired fruit and seed formation. In rice seedlings, imidacloprid and chlorpyrifos were reported to reduce the root length, shoot length, fresh weight and protein content. These pesticides were also reported to degrade the chlorophyll pigments (Sharma *et al*., 2012, 2013). Sharma *et al*. (2015) reported that imidacloprid and chlorpyrifos cause oxidative stress to rice seedlings by generating reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide (H_2O_2). In tomato plants, Zhou *et al*. (2015) also reported the enhanced levels of H_2O_2 after the application of chlorothalonil pesticide. In cucumber plants, phytotoxic effects of various pesticides were studied by Xia *et al*. (2006). They observed that application of pesticides negatively affects the photosynthetic machinery of cucumber plants, resulting in reduced photosynthetic rate (Pn), stomatal conductance (Gs) and intercellular CO_2

(Ci). These researchers also reported the inhibitory effects of pesticides on quantum efficiency (F_v/F_m) of PS II. Siddiqi and Ahamad (2006), studied the effect of six different pesticides (topsin-M, demacron, benlate, cypermethrin, dimethride and chlorosulfuron) on soybean plants. They found that at higher concentrations (0.5 and 0.75 g L^{-1}), these pesticides decline relative growth rate and crop growth rate. Reduced total phenol and ascorbic acid contents in potatos were observed after the application of IMI (Chauhan *et al*., 2013). Application of pesticides (mancozeb, flusilazol, dithianon, pirimicarb) on apple trees was reported to inhibit the process of photosynthesis (Untiedt and Blanke, 2004). In *Saccharina japonica,* application of diuron pesticide resulted in retarded growth, decreased carotenoid content, chlorophyll content, optimal quantum yield and maximum electron transport rate (Kumar *et al*., 2010).

Pesticides are detoxified by plants or other environmental agents resulting in decrease in residues in plant products, although a continuous exposure to pesticide residues may cause deadly diseases like cancer (Carrozza *et al*., 2009). It is therefore imperative to estimate the residues of pesticides so that the agricultural products with least pesticide content will be available to the consumers (Ko *et al*., 2014). After translocation to aerial parts of the plant, IMI gets accumulated in plant parts, with higher concentrations of residues being in early leaves followed by young leaves, flowers and fruits. Roots accumulate very less amounts of IMI residues (Laurent and Rathahao, 2003; Alsayeda *et al*., 2008). Accumulated pesticide is then detoxified by the plant via three step enzymatic detoxification system. In the first step, pesticides are activated by enzymes like P450 monooxygenases, peroxidases and carboxylesterases. In the second step of pesticide metabolism, glutathione-s-transferase (GST) and UDP-glycosyltransferase help in conjugation to glutathione and glucose. Third step involves the isolation and deposition of soluble metabolites in cell organelles like vacuoles and apoplast (Coleman *et al*., 1997; Cherian and Oliveira, 2005). In plants IMI is reported to degrade into 6-chloronicotinic acid, 6-chloropicolyl alcohol, guanidine, nitrosamine, urea imidacloprid, 5-hydroxy and olefine metabolites (Sur and Stork, 2003; Thurman *et al*., 2013). Environmental protection agency (EPA, 2014) established tolerance for residues of IMI and its metabolites which includes 0.05 mg Kg^{-1} in Indian mustard seeds and 3.5 mg Kg^{-1} in leafy vegetables of *Brassica* spp.

1.3 Brassinosteroids and their ability to ameliorate pesticide toxicity

Brassinosteroids (BRs) belong to plant polyhydroxysteroids and occur in plants in small concentrations in pollens, seeds, and young vegetative tissues (Clouse and Sasse, 1998; Bhardwaj *et al*., 2008; Kanwar *et al*., 2015). They play an important role in stress protection in plants caused by various abiotic factors like temperature, salt, drought, ozone, pesticides, herbicides and heavy metals (Krishna, 2003; Sharma *et al*., 2012, 2013, 2015). As a consequence of pesticide toxicity, plant growth and development are negatively affected due to generation of reactive oxygen species. However, in response to this pesticide stress, the plant's internal defense system (antioxidative defense system) gets activated to cope up with pesticide toxicity. Moreover, BR application further triggers this antioxidative defense system of the plants, resulting in enhancing resistance of plants to pesticide toxicity. 24-epibrassinolide (EBR) is one of the most bioactive forms of BRs which are mostly used in experimental studies (Vardhini and Anjum, 2015). EBR is white, crystalline in nature having chemical formula of $C_{28}H_{48}O_6$ with molecular weight of 480.68 g mol^{-1}.

In cucumber plants, exogenous application of EBR increases photosynthetic rate and stomatal conductance, which were earlier negatively affected by pesticide application (Xia *et al*., 2006). These investigators reported that application of 0.48 g L^{-1} chlorpyrifos decreased photosynthetic rate and stomatal conductance by 81.0 and 71.9 % respectively when compared to the control plants. However, application of EBR enhanced photosynthetic rate and stomatal conductance by 395 and 277% respectively when compared to the plants treated with chlorpyrifos only. These researchers also observed that application of EBR significantly increased the quantum efficiency of PSII and phytochemical quenching co-efficient. Antioxidative defense system of plants gets activated under pesticide stress (Xia *et al*., 2009a; Sharma *et al*., 2012, 2013, 2015; Zhou *et al*., 2015). EBR was reported to enhance the activities of antioxidative enzymes like superoxide dismutase, catalase, ascorbate peroxidase (POD), glutathione peroxidase, glutathione reductase (GR), dehydroascorbate reductase, monodehydroascorbate reductase, and contents of protein, proline, under chlorpyrifos (CPF) and IMI pesticide stress in rice seedlings (Sharma *et al*., 2012, 2013). They also noticed the stimulatory effect of EBR on overall growth of rice seedlings under CPF

and IMI toxicity. Non-enzymatic antioxidants like ascorbic acid, tocopherol, glutathione, polyphenols and total phenolics are components of plant antioxidative defense system, which get activated under abiotic stress conditions (Vardhini and Anjum, 2015). The contents of all these antioxidants were reported to increase under pesticide, salt and heavy metal stress conditions (Siddiqui and Ahmed, 2006; El-Mashad and Mohamed, 2012; Kapoor *et al*., 2014). BRs enhance total phenolics, ascorbic acid, tocopherol and glutathione contents in plants under normal as well as salt and pesticide stressed conditions (El-Mashad and Mohamed, 2012; Serna *et al*., 2013; Champa *et al*., 2015; Zhou *et al*., 2015).

Expression and activities of enzymes involved in enzyme mediated pesticide detoxification system were reported to be enhanced after the application of BRs (Xia *et al*., 2009a; Zhou *et al*., 2015). In pesticide stress protection, BRs are directly involved in degrading the pesticides, thus holding strong future prospects (Kang and Guo, 2011). Xia *et al*. (2009a) found that, EBR application to cucumber plants reduced the pesticide residues (chlorpyrifos, carbendazim, cypermethrin and chlorothalonil) by more than 30%. They further reported that reduced pesticide residues were accompanied by the enhanced activity of antioxidative enzymes including POD, GST, and GR. These researchers also observed that exogenous application of EBR significantly enhanced the expression of *P450* (P450 monooxygenase), *GST* and *MRP* (Multidrug resistance associated protein) genes which are responsible for pesticide detoxification in plants. BRs triggered the pesticide degradation in intact plants by 34 to 71% (chlorpyrifos in cucumber, tea, rice, broccoli and chinese cabbage; phoxim in tea and chinese chives; chlorothalonil in tomato, celery, strawberry and asparagus; omethoate in cucumber; cypermethrin in cucumber, tea, chinese cabbage and broccoli; carbofuran in garlic and chinese chives; and 3-hydroxycarbofuran in chinese chives) (Zhou *et al*., 2015). They also reported that EBR enhanced the expression of genes under chlorothalonil pesticide stress in tomato plants. They further noticed that a total of 1725 genes were expressed when EBR was given along with chlorothalonil pesticide. However, in alone EBR treated plants, 1584 genes and in alone chlorothalonil treated plants, 1545 genes were expressed. They also observed that out of all the expressed genes, 301 were mutually up-regulated by all the three treatments. Recently, it has been reported that mitogen

activated protein kinase (MAPK) and nitric oxide (NO) play an important role in BRs mediated pesticide detoxification (Yin *et al*., 2016).

1.4 Aims and objectives

Keeping in mind the protective roles of EBR against pesticide toxicity in plants, the present study was designed to meet the below mentioned objectives.

1. To study the effects of EBR on growth parameters of *B. juncea* plants grown under IMI toxicity.
2. Effects of EBR on pigments and gaseous exchange systems of *B. juncea* plants exposed under IMI toxicity.
3. Antioxidative defense system of *B. juncea* plants given binary treatments with IMI and EBR.
4. Influence of EBR on amino acid, protein and organic acid metabolism in *B. juncea* plants grown under IMI toxicity.
5. Impact of EBR on elemental and phytochemical composition of *B. juncea* plants grown in Petri-plates/soils amended with IMI.
6. EBR modulated gene expression and IMI residues in *B. juncea* plants grown in IMI amended medium.

Though the use of pesticides in any form should be discouraged, or these be used minimally, the present study attempts to find the possibility of EBR seed soaking to mitigate the toxic effects of IMI on *B. juncea*. The study in its modest will also help to understand the mechanism of interaction between EBR and IMI with special reference to physiological, biochemical and molecular aspects.