CHAPTER 7

Role of Gibberellin, Ethrel and Silverthiosulfate in abscission of flowers in *Jatropha curcas*

Role of Gibberellin, Ethrel and Silverthiosulfate in abscission of flowers in *Jatropha curcas*

7.1. Introduction

Phytohormones have vital role in assimilating many aspects of plant growth and development. Regulation of hormonal signals can occur via fluxes in the level of the hormone or in its perception (Bradford and Trewavas, 1994). Sensitivity to hormones can be regulated both spatially and temporally. For example, adjacent cells can respond differentially to a hormone, as can occur during abscission. Abscission is a physiological process that involves the programmed separation of entire organ, such as leaves, petals, flowers, and fruit. Abscission allows plants to discard nonfunctional or infected organs, and promotes dispersion of progeny. Abscission occurs due to various reasons. For eg., in Fruit drop, the abscission of fruit while still immature, is a naturally occurring process. Abscission is seen after the formation of an abscission zone at the point of separation. Within this zone, a thin layer of cells, the abscission layer, becomes weakened and breaks down through the conversion of pectic acid to pectin. As a result of this, the leaf, fruit, or other part can easily be dislodged by wind or rain. The process is thought to be controlled by the amount of auxin present. At the cellular level, abscission is the hydrolysis of the middle lamella of an anatomically specialized cell layer, the abscission zone (AZ), by cell wall-modifying and hydrolyzing enzymes. Thus, abscission requires both the formation of the AZ early in the development of a plant organ and the subsequent activation of the cell separation response (Bleecker et al., 1997; Patterson, 2001; Roberts et al., 2002; Lewis et al., 2006). Studies on *Arabidopsis thaliana* have implicated the involvement of several different genes in the control of abscission including potential signal molecules, receptors and other gene products (Lewis et al., 2006). *HAESA* (*HAE*), one of the first *Arabidopsis* receptor-like protein kinases (RLK) identified, is shown to be expressed in floral organ AZ (Jinn et al., 2000).

Organ abscission is believed to be a highly coordinated process where the plant hormone ethylene also plays an important role in regulating the breakdown of cell wall

of a layer of cells in the AZ. Abscission, senescence, and ripening are plant developmental processes whose timing is determined by tissue sensitivity to ethylene (Trewavas, 1986; Bleecker and Patterson, 1997; Zegzouti et al., 1999). The biological basis for this increased ethylene sensitivity is still not known, but it has been shown to be modulated also by other plant hormones. In abscission, the interplay between indole-3-acetic acid (IAA) and ethylene is well-established (Abeles and Rubinstein, 1964; Sexton, 1997; Taylor and Whitelaw, 2001; Roberts et al., 2002). Ethylene is a clear regulator of plant senescence in some species (Stead and Van Doorn, 1994). Ethylene is involved in the natural senescence of only a minority of the wilting type of flowers and in a majority (if not all) of the abscising type of flowers. Ethylene biosynthetic genes induce the production of ethylene during senescence. Ethylene plays a regulatory role in the release of ROS during PCD. It is well documented that during leaf, flower and fruit abscission cell wall degradation is associated with an increase in the activity of several hydrolytic enzymes (Bethke et al., 2003). PCD in wilting of flowers leads to disappearance of most of the organelles and accumulation of degrading enzymes in the vacuole. This leads to DNA degradation, chromatin condensation, and a decrease in nuclear diameter, parameters that indicate PCD. The timing of PCD and abscission may be regulated independently. In many species, PCD occurs much earlier than abscission, whereas in other species abscission comes first. The initial symptom of senescence was either wilting or abscission, but in some species, the time span between wilting and abscission was very short.

The use of terms senescence and programmed cell death have led to some confusion. Senescence as visibly observed in, for example, leaf yellowing and petal wilting, has often been taken to be synonymous with PCD of the constituent cells. The mechanisms of PCD in plants bear a certain relation to those of apoptosis, and to some processes, such as nucleic acid degradation, which are superficially similar to some aspects of the senescence syndrome. PCD also refers to changes in cells, which follow a program leading to their death. PCD is an inbuilt suicide program that is induced to ensure that organisms show appropriate developmental patterns and growth responses to external challenges. Vacuolar integrity typically plays a central role in controlling the initiation and progress of PCD, as rupture of the tonoplast results in the release of

hydrolytic enzymes stored in the vacuole, leading to rapid degradation of the various organelles and nuclear DNA (Kuriyama, 1999; Obara et al., 2001).

Developmental PCD in plants occurs, for instance, during embryogenesis (Bozhkov et al., 2005), leaf morphogenesis (Gunawardena, 2007), floral development (Rogers, 2006) and organ senescence (Rogers, 2005). Senescence is an organ-level phenomenon. Senescence as a type of metaplasia includes a protective role for the vacuole during transdifferentiation, followed by an autolytic function, which terminates senescence and cauterizes the tissue. Cysteine proteases are the closest functional homologue to caspases in senescing plant tissues and these are commonly found during leaf and petal senescence (Buchanan-Wollaston, 1997; Griffiths et al., 1997; Wagstaff et al., 2002). Senescence in cells is the same as PCD and the two are fully synchronous. The death of plant cells is preceded by a loss of membrane permeability. This is due in part to increases in reactive oxygen species that are in turn related to up-regulation of oxidative enzyme and to a decrease in activity of certain protective enzymes. There is little evidence that the molecular and biochemical events responsible for the altered morphology leading to cell death are identical in plant and animal systems. In fact, it is likely that plant cells, surrounded by complex cell walls, containing large central vacuoles, have unique mechanisms for bringing about cell death. During senescence or abscsssion, Catalase (cat) lowers H_2O_2 level, by converting H_2O_2 to H_2O and O_2 (Scandalius, 1993). Overexpression of the gene (cat) for this enzyme protects leaves against ROS (Zelitch et al., 1991) and CAT-deficient plants are more sensitive to a variety of stresses (Willekins et al., 1997). When senescence is induced, Catalase activity is the first to decrease (Panavas and Rubinstein, 1998). SOD protects the plants against buildup of ROS. As the product of SOD is H_2O_2, which is also an ROS, if an effective scavenging system is damaged it will result in cell death. Studies on abscission in daylily have shown an increase in SOD activity resulting in cell death (Panavas and Rubinstein, 1998). Peroxidase activity uses H_2O_2 as a substrate for several reactions and its specific activity increases in both carnation (Bartoli et al., 1995) and daylily (Panavas and Rubinstein, 1998) during abscission. These reports suggest that Peroxidase activity is a key player in responding to conditions of oxidative stress that result from H_2O_2 accumulations.

In previous chapters (4 and 5), we have shown that increased GA treatment increases flowering and femaleness. We also observed that, these plants had increased peduncle length, which was more so in female flowers. The increase in peduncle length led to a weaker peduncle causing abscission of flower before pollination. This resulted in poor seed yield inspite of a favourable Male: Female ratio. There are various reports that show that abscission is the consequence of formation of AZ. Our results also show that increased exposure to Ethylene and Silver thiosulfate treatments shows withering of flowers before maturation. Thus, in this chapter, we discuss the role of phytohormones in bringing about abscission of flower and fruit and the changes that occur leading to the formation of AZ. A correlation between ROS, DNA fragmentation and cell death during abscission caused by phytohormones is discussed.

7.2. Results

7.2.1. Effect of GA, Ethrel and Silver thiosulfate on abscission of Inflorescence

Phytohormones GA and Ethrel and Silver thiosulfate were sprayed on emerging floral bud. Its effect on inflorescence at stage V was observed. Photographs were captured when the flowers were in stage V of inflorescence. Results are representative of three independent experiments.

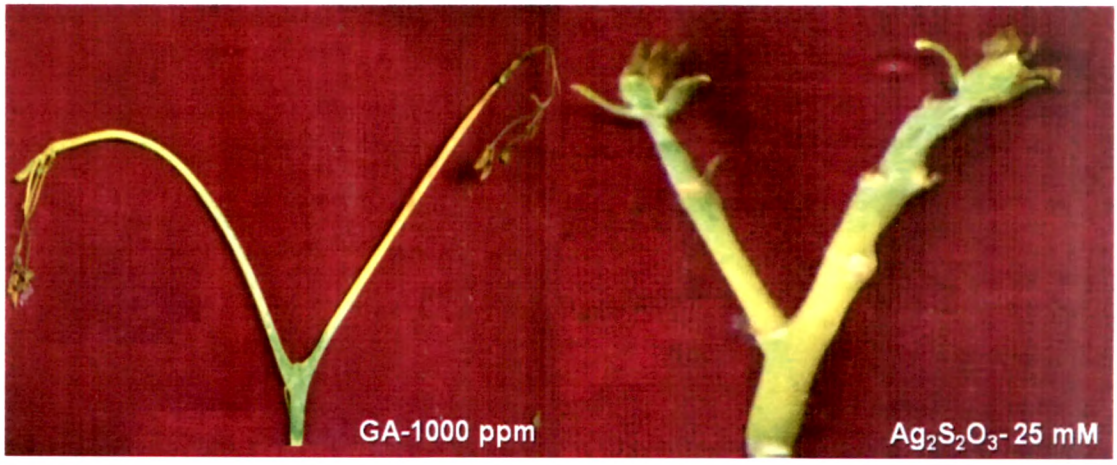

Figure 7.1 (a): Deleterious effect of GA and Silver thiosulfate on Inflorescence morphology

Figure 7.1 (a) shows how GA (1000 ppm) tremendously increases the length of peduncle (especially that of female flowers) compared to other treatments. It leads to weaker, thin and elongated peduncle causing abscission due to abscission zone formation. Silver thiosulfate (25 mM) also led to withering of flowers.

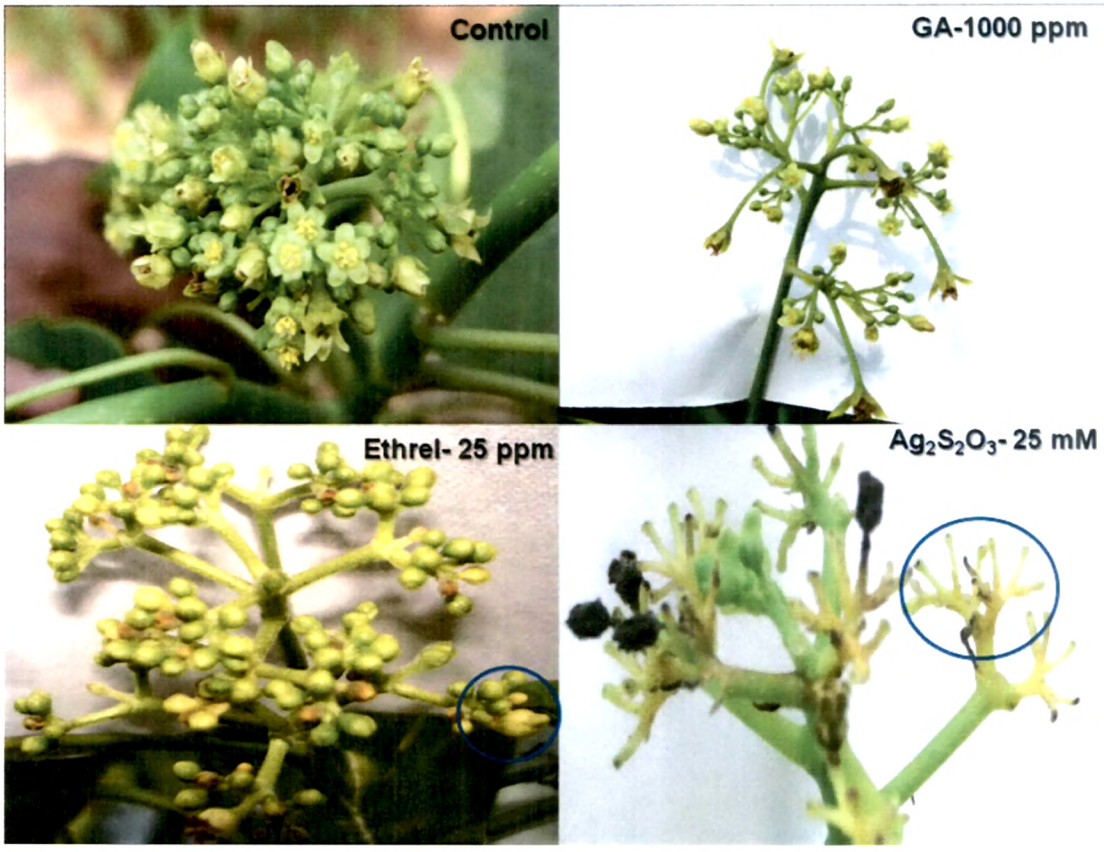

Figure 7.1 (b): Effect of GA, Ethrel and Silver thiosulfate on Inflorescence morphology

As shown in above figure 7.1 (b), GA (1000 ppm) treatment increases the length of peduncle especially in female flower. Treatment with Ethrel (25 ppm) causes most of female floral bud to brown and leading to early abscission (as highlighted). Silver thiosulfate also caused increase abscission of most of flowers before it goes to maturation. This effect was the same for male or female flowers.

7.2.2 Biochemical changes seen due to GA, Ethrel and Silver thiosulfate treatment at abscission zone in *Jatropha curcas*

The treatment of GA, Ethrel and Silver thiosulfate on developing flowers caused abscission. The biochemical changes occurring at AZ were studied to understand the mechanism of abscission. As it is well documented that hydrolytic changes precede AZ formation the levels of Hydrogen peroxide, Catalase, SOD and Peroxidase activity were monitored.

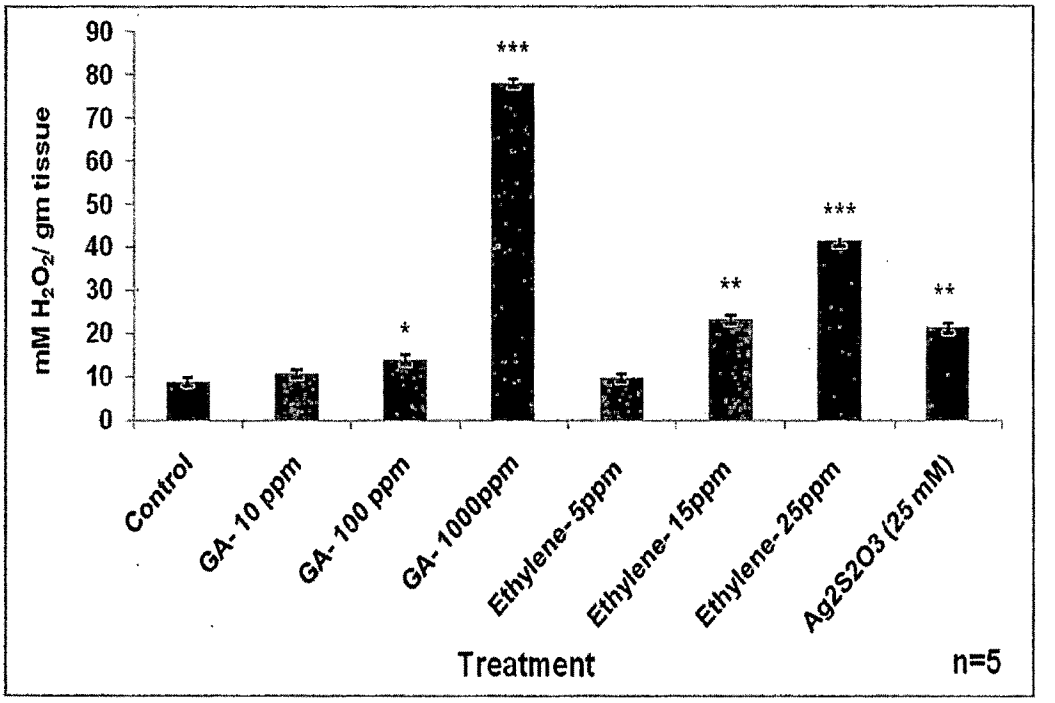

Figure 7.2: Effect of GA, Ethrel and Silver thiosulfate on Endogenous hydrogen peroxide

Values are mean ± SE; *, **, * indicates significantly different at $p<0.05$, $p<0.01$ and $p<0.001$ respectively as compared to the corresponding control.**

Hydrogen peroxide was determined by Zhou et al., (2006) as mentioned in Materials and Methods section. The levels of Hydrogen peroxide in the plants treated with different phytohormones are shown in figure 7.2. H_2O_2 concentration increased with increase in exposure to GA and Ethrel. Silver thiosulfate (25 mM) treatment also significantly increased the level of H_2O_2 compared to control.

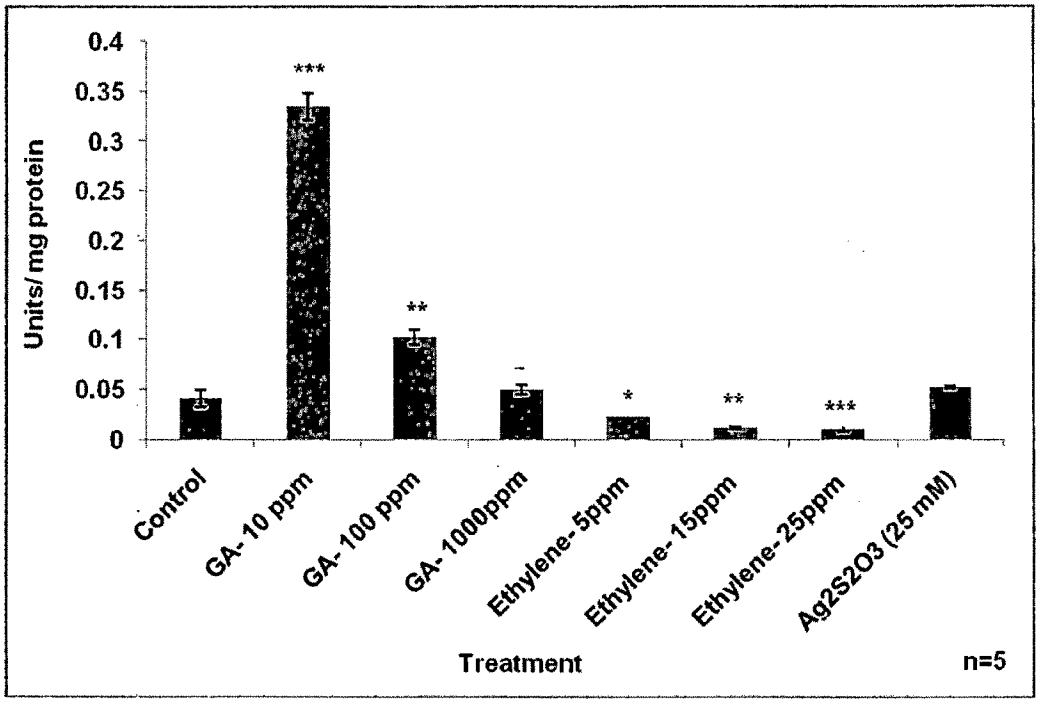

Figure 7.3: Effect of GA, Ethrel and Silver thiosulfate on Catalase activity

Values are mean ± SE; *, **, * indicates significantly different at p<0.05, p<0.01 and p<0.001 respectively as compared to the corresponding control.**

Catalase activity was determined by Aebi et al., (1984) as mentioned in Materials and Methods section. Catalase activity was significantly decreased with increase in concentration of GA and Ethrel (Figure 7.3). Silver thiosulfate effect shows no significant change in catalase activity compared to control.

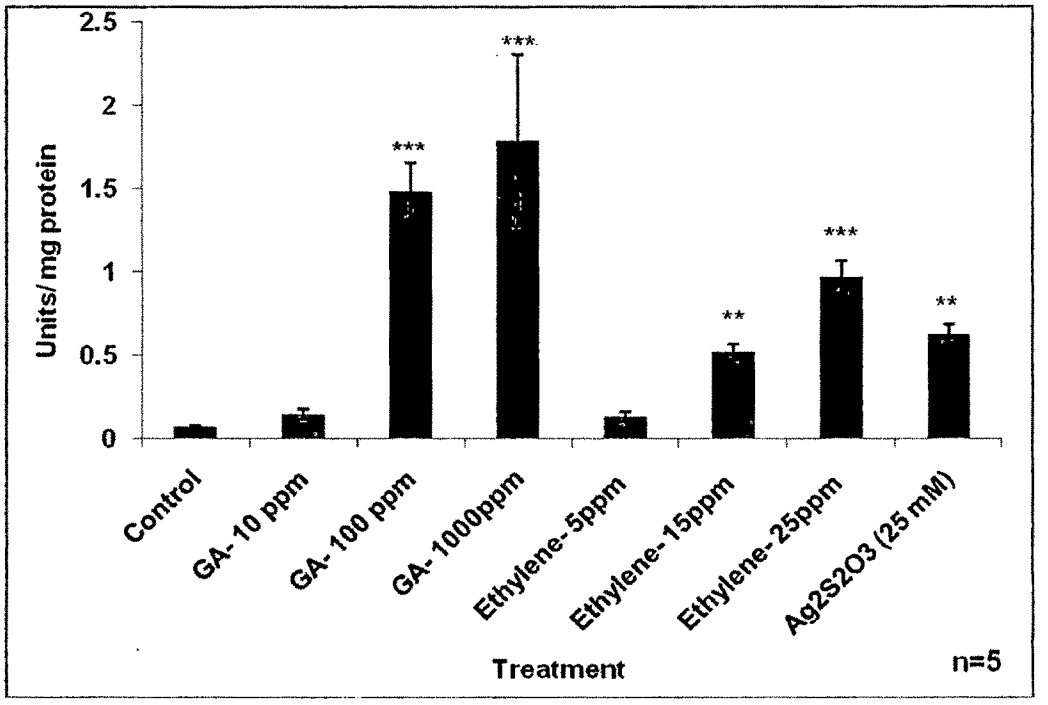

Figure 7.4: Effect of GA, Ethrel and Silver thiosulfate on Peroxidase activity

Values are mean ± SE; **, * indicates significantly different at p<0.01 and p<0.001 respectively as compared to the corresponding control.**

Peroxidase activity was determined by Chance and Maehly, (1955) as mentioned in Materials and Methods (Chapter 2). Peroxidase activity was significantly higher and increased with increase in concentration of GA and Ethrel treatments as shown in figure 7.4. Silver thiosulfate treatment significantly increased the Peroxidase activity as compared to control.

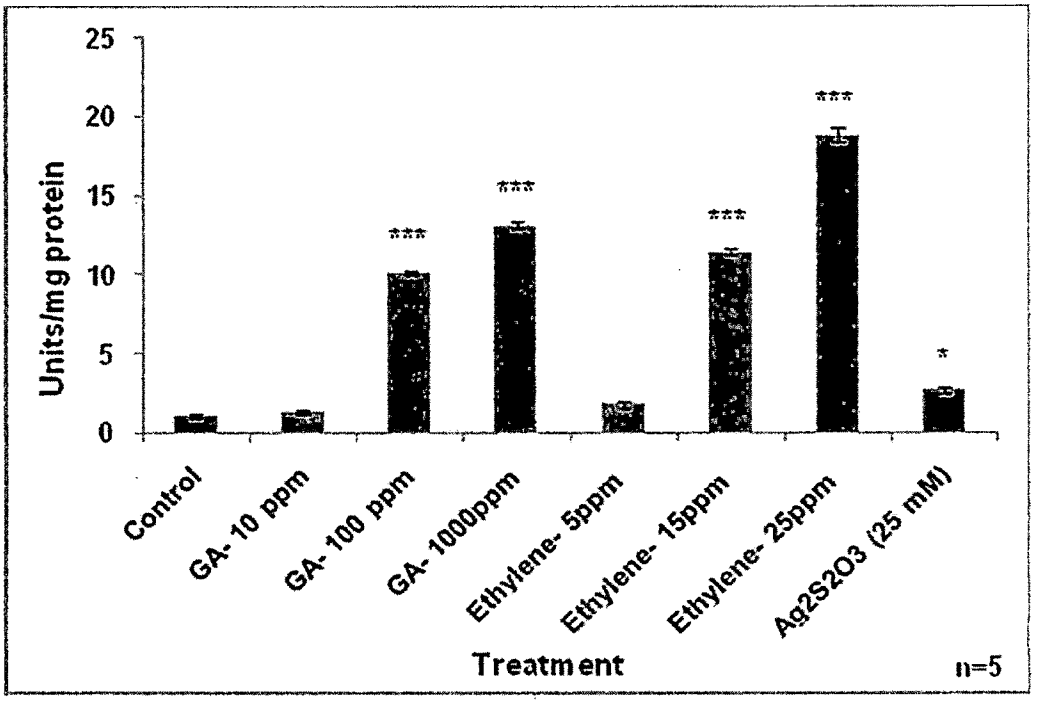

Figure 7.5: Effect of GA, Ethrel and Silver thiosulfate on SOD activity

Values are mean ± SE; *, * indicates significantly different at p<0.01 and p<0.001 respectively as compared to the corresponding control.**

SOD activity is determined by Marklund and Marklund (1984) method as mentioned in Chapter 2. SOD activity was significantly increased with increase in concentration of GA and Ethrel treatment as shown in figure 7.5. Silver thiosulfate (25 mM) treatment significantly increases the SOD activity as compared to control.

7.2.3. Effect of GA, Ethrel and Silver thiosulfate on Cell viability

Histological study of the abscission zone was done to observe the cells. Tissue was dissected and made into thin sections. The sections were stained in Evans blue stain to differentiate between the live and dead cells according to the protocol mentioned in chapter 2.

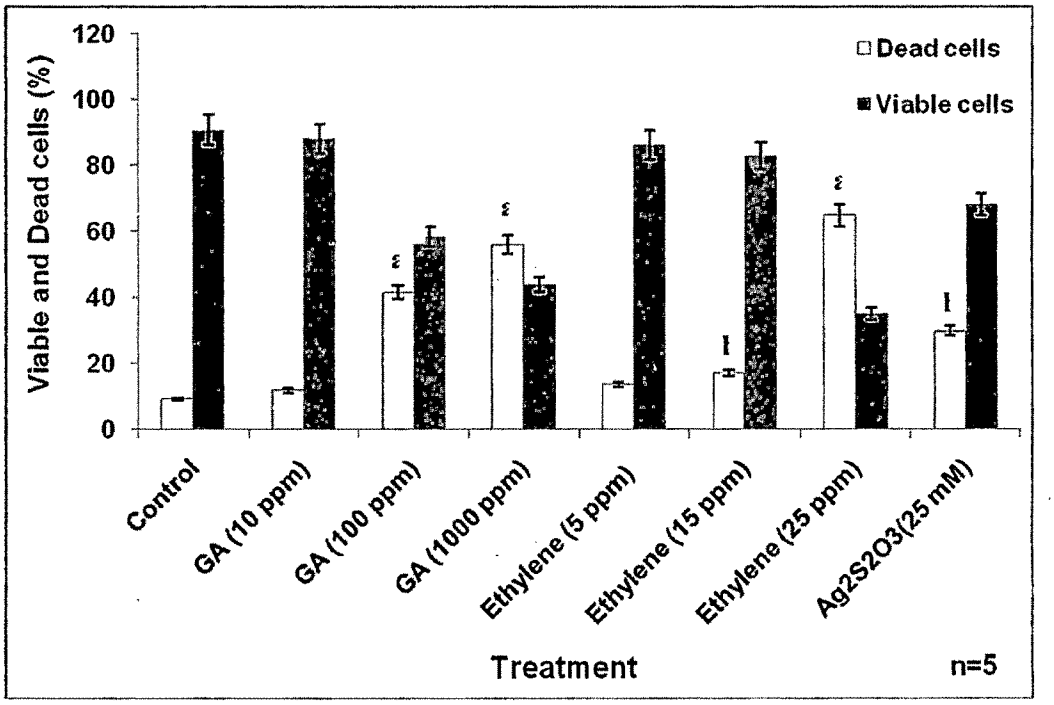

Figure 7.6: Effect of GA, Ethrel and Silver thiosulfate on Viability of cells

Values are mean ± SE; 'a' and 'b' indicates significantly different at p<0.001 and p<0.01 respectively as compared to the corresponding control.

The number of dead cells were significantly higher in the abscission zone of the plants treated with higher concentration of GA and Ethrel as shown in figure 7.6. Hence it can be concluded that cell viability is decreased with increased concentration of GA and Ethrel treatments. Silver thiosulfate also significantly increased the number dead cells as compared to control.

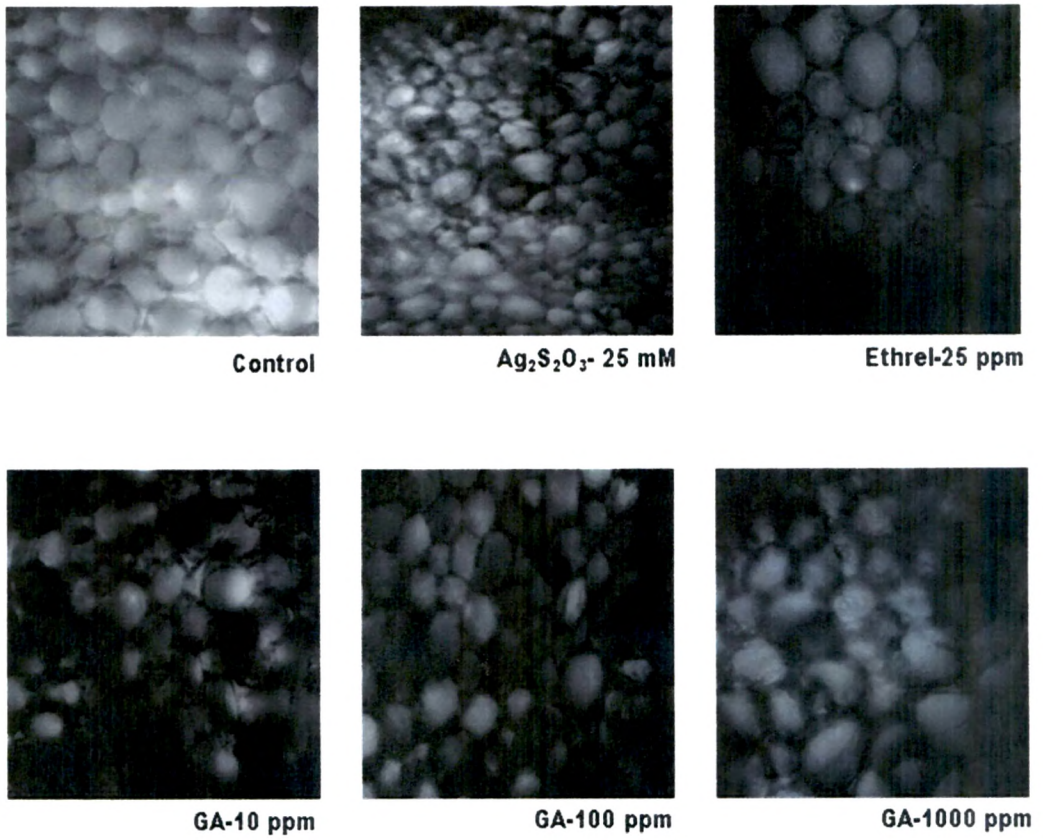

Figure 7.7: Monitoring cell viability Trypan blue exclusion method

There is considerable tissue damage at the abscission zone after GA, Ethrel and Silver thiosulfate treatments as compared to control (Figure 7.7). Thin section of stained tissue were observed under microscope at 4X magnification. There is complete tissue damage on GA (1000 ppm) treatment. Ethrel and Silver thiosulfate also has a similar affect. Results are representative of three independent experiments.

7.2.4. Effect of GA, Ethrel and Silver thiosulfate on DNA fragmentation by DAPI and Gel electrophoresis

DNA was isolated from abscission region after GA, Ethrel and Silver thiosulfate treatments. DNA was run on 1% agarose gel, method as mentioned in chapter 2.

Figure 7.8: Monitoring DNA fragmentation by Gel electrophoresis

GA (1000 ppm) treatment shows complete damage of DNA as compared to control and other treatments. Lower dose of GA, Ethrel and Silver thiosulfate have no effect on DNA as shown in figure. GA (1000 ppm) treatment lead to DNA smearing as shown in figure 7.8.

Nuclear morphology of cells in the tissue from the Abscission zone was studied using DAPI dye. The tissue was prepared as mentioned in Materials and Method (Chapter 2). Cells were stained with DAPI dye and observed under Bright field

Microscope with 20X magnification and afterwards the same field was observed under flourescence lamp.

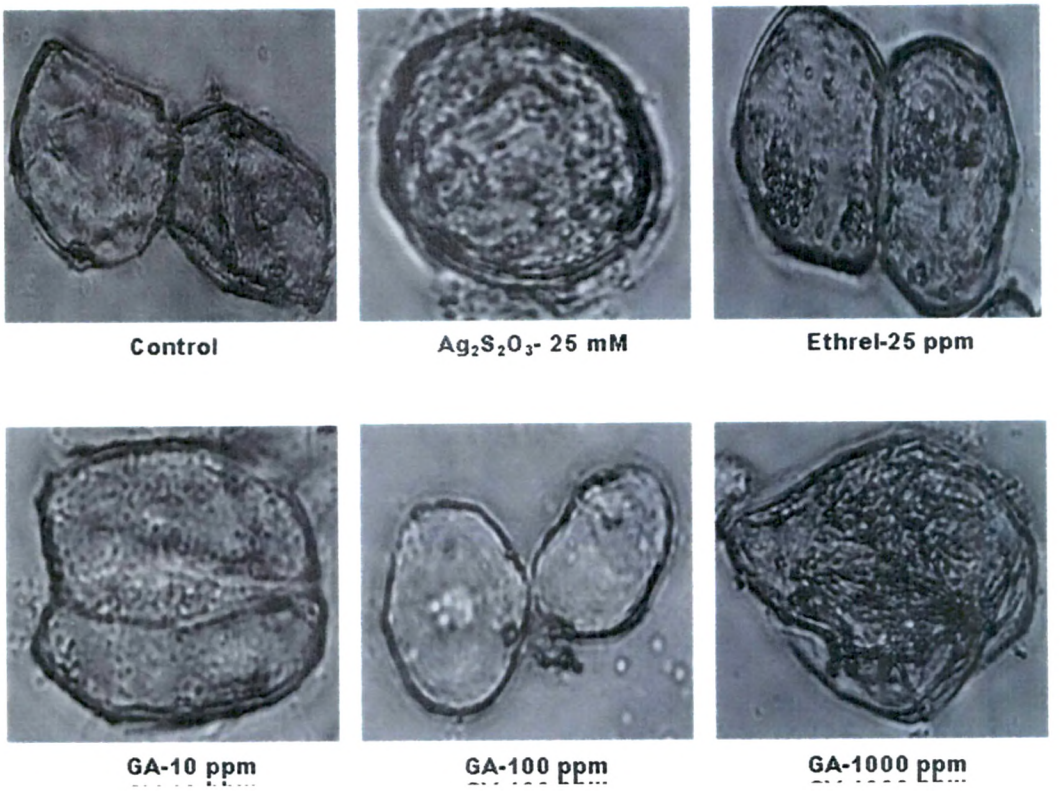

Figure 7.9: Cell morphology monitored by Bright field microscopy

GA (1000 ppm) treatment leads to complete damage of cells as compared to control and other treatments. GA (100 ppm), Ethrel (25 ppm) and Silver thiosulfate (25 mM) treatments changed the cell shape and morphology. It led to vacuolation and membrane impermeability as compared to control (Figure 7.9). Photographs were captured at 20X magnification. Results are representative of three independent experiments.

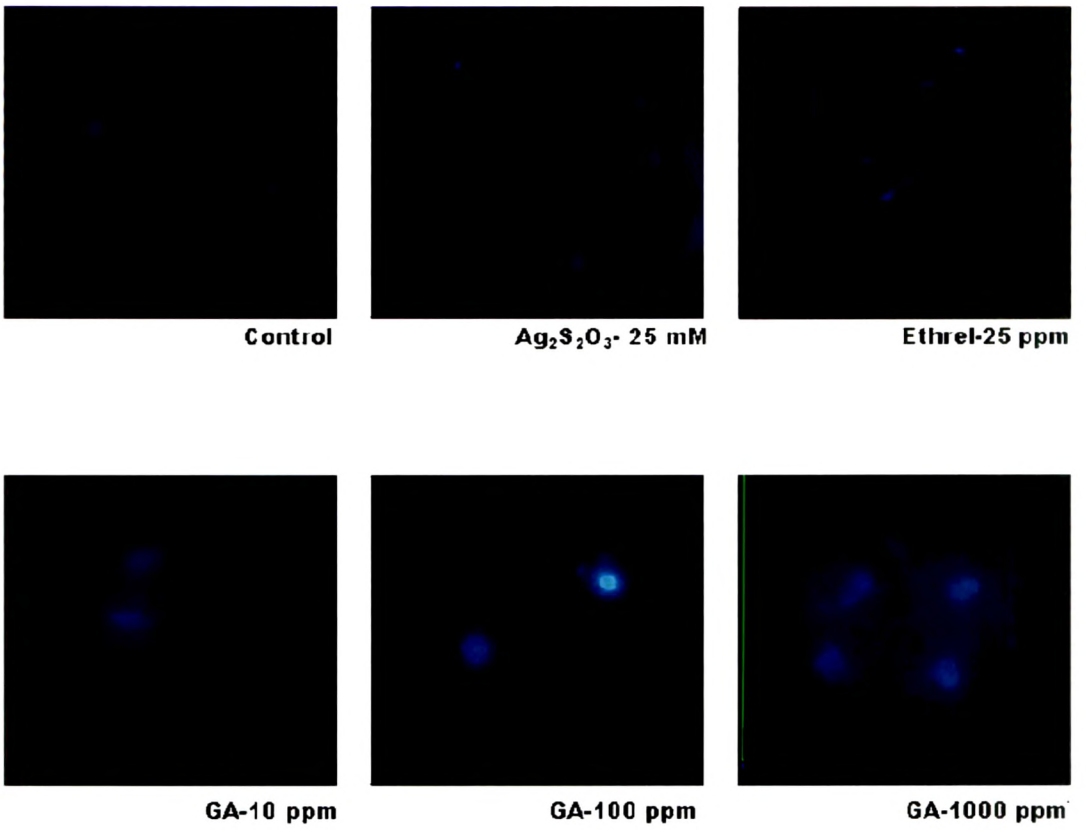

Figure 7.10: Nuclear morphology monitored by DAPI staining

As shown in figure 7.10, Increased GA treatment shows high contrast and condensation of nuclei than control and other treatments. GA (1000 ppm) shows complete damage and dispersion of nuclei and nuclear membrane indicating necrotic type of cell death.

7.3. Discussion

7.3.1. Effect of GA, Ethrel and Silver thiosulfate on abscission of Inflorescence

GA (1000) treatment increases the withering of inflorescences due to an increase in the length of peduncle and formation of abscission zone. DELLA protein has been shown to be involved in mediating senescence via inhibition of GA action in *Arabidopsis thaliana* (Achard et al., 2007). Ethylene at higher dose also leads to abscission. Ethylene is the major co-ordinator of abscission in many flowers (Abeles et al., 1992; Wagstaff et

al., 2005). The deterioration in condition at the abscission zone of the flowers was hastened by exogenous ethylene. Senescence is also accompanied by increased endogenous ethylene biosynthesis (Nichols, 1977). Higher GA and Ethrel treatment leads to increased endogenous level of ethylene which is responsible for abscission zone formation and hence senescence. This is supported, by the result in chapter 6, which shows that GA and Ethrel treatment increases the endogenous level of GA and Ethylene and decreases the endogenous level of IAA. Ethylene induced leaf abscission is promoted by GA, a fact that has been documented by several other scientists (Morgan et al., 1975; Steffens and Sauter, 2005). Silver thiosulphate was used as an inhibitor of Ethylene release, however in our studies it resulted in flower senescence and cell death instead of reversing ethylene effects. This could be because of the concentration used. However, there is a report, which substantiates our observations where they have shown Silver thiosulfate application causing premature cell death in *Pythium ultimum* (Hausbeck, 1989).

7.3.2 Biochemical changes seen due to GA, Ethrel and Silver thiosuflate treatment at abscission zone in *Jatropha curcas*

Because of alteration in various parameters involved in oxidative reactions, ROS is generated, which could be responsible for death of plant cells including petals, leaves, flowers and fruits. The ROS produced from hydrogen peroxide regulates enzymes involved in abscission (Halliwell and Gutteridge, 1989). Increased GA and Ethrel treatment increases SOD, Peroxidase activity, decreases Catalase and leads to accumulation of Hydrogen peroxide in abscission zone. In daylily, an increase in ROS decreases the activity of Catalase and increased activity of Peroxidase, SOD (Panavas and Rubinstein, 1998). In carnation petals, Catalase and Ascorbate peroxidase activities are increased during flower senescence (Bartoli et al., 1995). An increase in the number of peroxisomes during carnation petal senescence has also been reported. Jong et al., 2002 also reported that ethylene releases hydrogen peroxide during PCD in tomato suspension cells. Sauter et al., 2009 has reported increased ethylene levels as being responsible for induction of H_2O_2 levels through H_2O_2 synthesis by NADPH. Silver thiosulfate is also responsible for increased H_2O_2 levels that lead to increase in SOD and

Peroxidase activity (Rostami et al., 2010). Thus, higher dose of GA, Ethrel and Silver thiosulfate leads to Abscission zone formation at flower base due to hydrogen peroxide release brought about by alteration in the activity of Catalase, SOD and Peroxidase leading. These changes trigger PCD.

7.3.3. Effect of GA, Ethrel and Silver thiosulfate on Cell viability

The changes in activities of enzymes regulating the antioxidant status of a cell determine its fate. ROS is one of the important factors responsible for Abscission and PCD (Schopfer et al., 2001; Heyno et al., 2011). Increased GA and Ethrel treatment has been reported to cause higher cell death due to the release of hydrogen peroxide leading to Abscission zone formation (Jong et al., 2002). Our results are in agreement with this observation. In addition, our results are similar for Silver thiosulfate treatment. The mechanism behind these observations needs elucidation.

7.3.4. Effect of GA, Ethrel and Silver thiosulfate on DNA fragmentation by Gel electrophoresis and nuclear morphology by DAPI

DNA cleavage is an early event in PCD. Gel electrophoresis indicates that DNA cleavage is random and does not result in bands that represent multiples of internucleosomal units. The dying cells display several hallmarks of PCD, including the early degradation of nuclear DNA, as indicated when stained with DAPI dye. The gel electrophoresis result suggests that GA (1000) treatment leads to necrosis of tissue as it shows DNA smearing. Increased GA treatment shows damage of nuclear envelope and nuclei. The nuclei showed chromatin condensation, as they showed brighter DNA fluorescence than control nuclei. The diameter of the condensed nuclei with condensed chromatin was different from that of control ones. The DNA degradation prior to cellular collapse seen in our results is similar to that reported, in *Iris petals* (Van der Kop et al., 2003; Van Doorn et al., 2003). The cell and its nuclear contents become degraded only after collapse of the vacuole (Obara et al. 2001). The condensation was seen to increase with increase in GA treatment as evident from an increase in fluorescence per unit surface, and from a decrease in nuclear size (Yamada et al., 2006). Ethrel (25 ppm)

treatment shows the chromatin condensation and DNA fluorescence. Ethylene is a potentiator of the oxidative burst and DNA damage in tomato suspension cells (Jong et al., 2002). Microscopy studies suggest that increased damaged of cell occurs as the GA treatment increases while Ethrel and Silver thiosulfate do not show any change in cell morphology. This indicates that nuclear damage is an early event, following which damage to cell structure is seen.