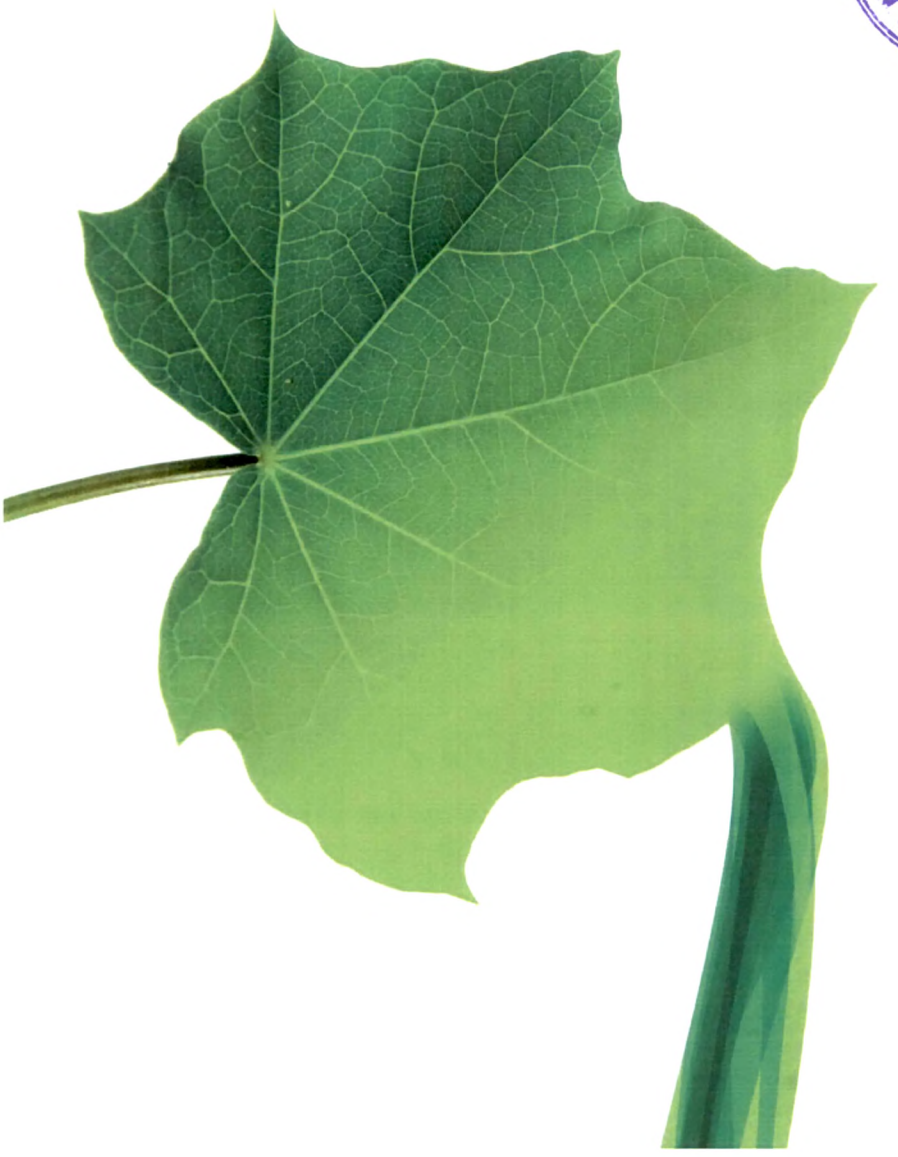
CHAPTER 6



Role of Gibberellin, 2, 4-D, Ethrel and Silverthiosulfate on endogenous levels of GA, Auxin, Ethylene and ACC in *Jatropha curcas*

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6.1. Introduction

In previous chapters, the effect of phytohormone on flower and fruit development, seed weight and oil content was studied. Historically, phytohormone signals were examined as individual pathways mediating a given response to a stimulus. Phytohormones are now recognized as functioning in complex signaling networks, often with interactive effects, referred to as crosstalk. The effect of these interactions has profound effect on plant responses, especially sex alteration. A large number of factors are responsible for regulation of phytohormone levels and hence, sex expression and sex alteration in plants (Dellaporta et al., 1994; Irish et al., 1989; Stehlik et al., 2008). In the results of the previous chapter, we have seen that GA at higher concentration and ethylene showed negative effect on femaleness. The regulation of ethylene biosynthesis during inflorescence development determines the sex of flower by regulating the expression of ACS in cucumber flower buds where it was shown that ACS mRNA is responsible for pistillate primordia. ACS is a key intermediate enzyme for ethylene biosynthesis. ACS mRNA accumulate just beneathe the pistil primordia of flower buds especially in the central region of the developing ovary where ovule and placenta form (Sayoko et al., 2007). GA deficiency shows retarded growth of all floral organs via DELLA regulation, resulting in abortive stamen development hence, complete male sterility (Hao Yu et al., 2004). DELLA proteins play a critical role in linking the GA signaling pathway with homeotic genes activity in flower development. However, Auxin has no direct role to regulate flowering homeotic genes but it indirectly promotes GA responses by destabilizing DELLA and by promoting the expression of GA biosynthetic genes (Frigerio et al., 2006; Fu and Harberd, 2003; O'Neill and Ross, 2002; Ross et al., 2000; Wolbang and Ross, 2001).

Phytohormone interaction was commonly considered at the level of signal transduction without proper consideration of phytohormone synthesis or accumulation

(Moller et al., 1999). The crosstalk of phytohormone and its effects could be studied by multiple phytohormone analyses. Numerous methods exist for the direct chemical quantification of individual phytohormones. Biochemical techniques to analyse IAA and GA involve time consuming protocols with multiple purification steps, and few target analytes. GC based analysis of chemical signals that includes metabolic profiling of key analytes can be used to study the effect of hormones (Weber et al., 1997; Muller et al., 2002; Engelberth et al., 2003). Towards this goal, in this study, we describe a simple method with broad potential applicability in the preparation and simultaneous GC analysis of the Ethylene and ACC. This readily available technology enables the exploration of interactions between physiologically and ecologically relevant chemical signals at the level of production; it also makes it possible to readdress previous findings in a comprehensive manner.

Although phytohormones, GA and IAA, were known to have essential role during flower growth and development, there is no inclusive study of their involvement in floral bud development (Hirano et al., 2008). They regulate cell expansion and tissue differentiation. Such reports exist for other physiological roles. GA stimulation during root elongation requires auxin (Weiss et al., 2007). It delays flower development via induction of cell elongation. Sometimes GA and Auxin have similar functions such as cell wall elongation but GA plays an important role in flowering. Its higher concentration in plant leads to abscission via abscission zone formation while higher concentration of Auxin inhibits abscission zone formation and delays the flower and fruit development. Thus, a physiological response seen is the net result of actual levels of phytohormones at that time point.

Hence, an attempt has been made to see the effect of GA, 2, 4-D, Ethrel and Silver thiosulfate on endogenous levels of the phytohormones and correlate it with changes in flowering and flower sex ratio. In this study, we analyzed the endogenous level of Indole-3-acetic acid (IAA), Gibberellin (GA), Ethylene and ACC at the time point of distinct appearance of floral bud.

6.2. Results

Tissue at specific stage of inflorescence (0.5 gm) i.e. distinct appearance of floral bud (Stage 2) was used for assaying the levels of phytohormones as per method described in Methods and Materials (Chapter 2) after the application of exogenous phytohormones.

6.2.1. Effect of Phytohormones on endogenous GA levels in floral bud during flower development

GA levels were determined by the method of Holbrook et al., 1961 as mentioned in Methods and Materials (Chapter 2). GA concentration was found to significantly increase with increase in concentration of exogenous treatment of phytohormones (Figure. 6.1). Silver thiosulfate treatment inhibits the level of GA. The level of GA significantly decreased when $Ag_2S_2O_3$ (25mM) was given alone or in combination with GA (1000 ppm). Of the Ethrel treatments, only Ethrel (25 ppm) brought about significant change in GA levels. 2, 4-D treatment had no significant effect on GA levels at lower concentration alone and with GA whereas GA with a higher dose expectedly increased GA levels.

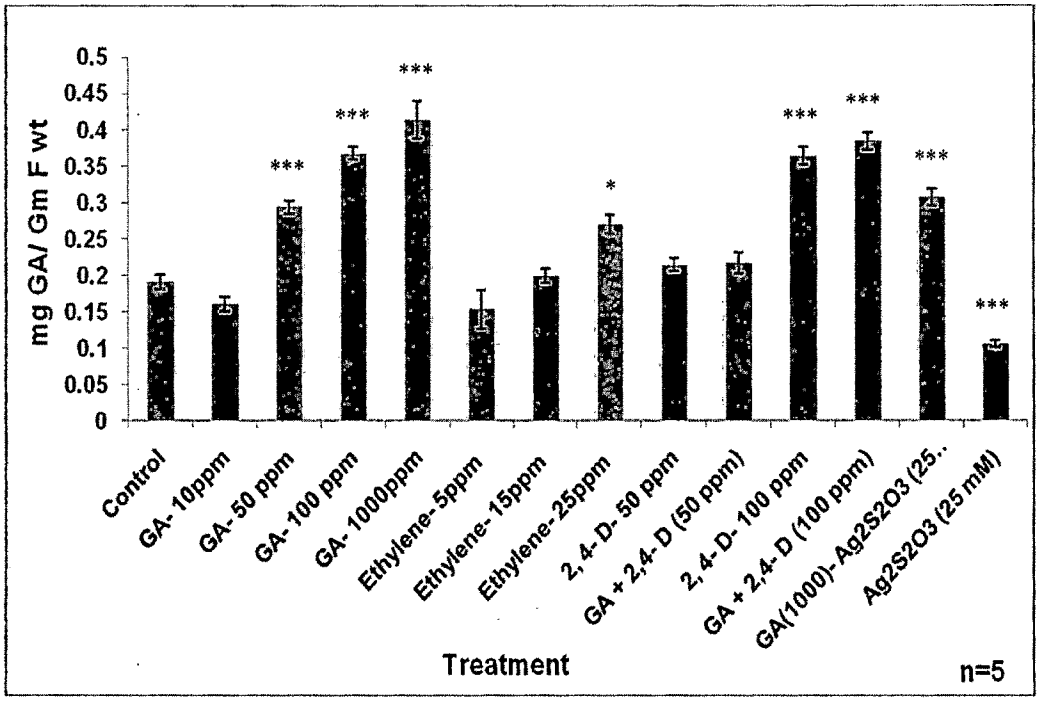

Figure 6.1: Effect of phytohormones on endogenous GA level in floral bud

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

6.2.2. Effect of Phytohormones on endogenous IAA level in floral bud during flower development

Auxin levels were determined by Gorden and Weber method (Gordon et al. 1951) modified as shown in the Methods and Material section. Auxin concentrations were significantly decreased with increase in concentration of GA and Ethylene treatments compared with control (Figure. 6.2). Silver thiosulfate treatment inhibits the release of endogenous Auxin alone or in combination with GA.

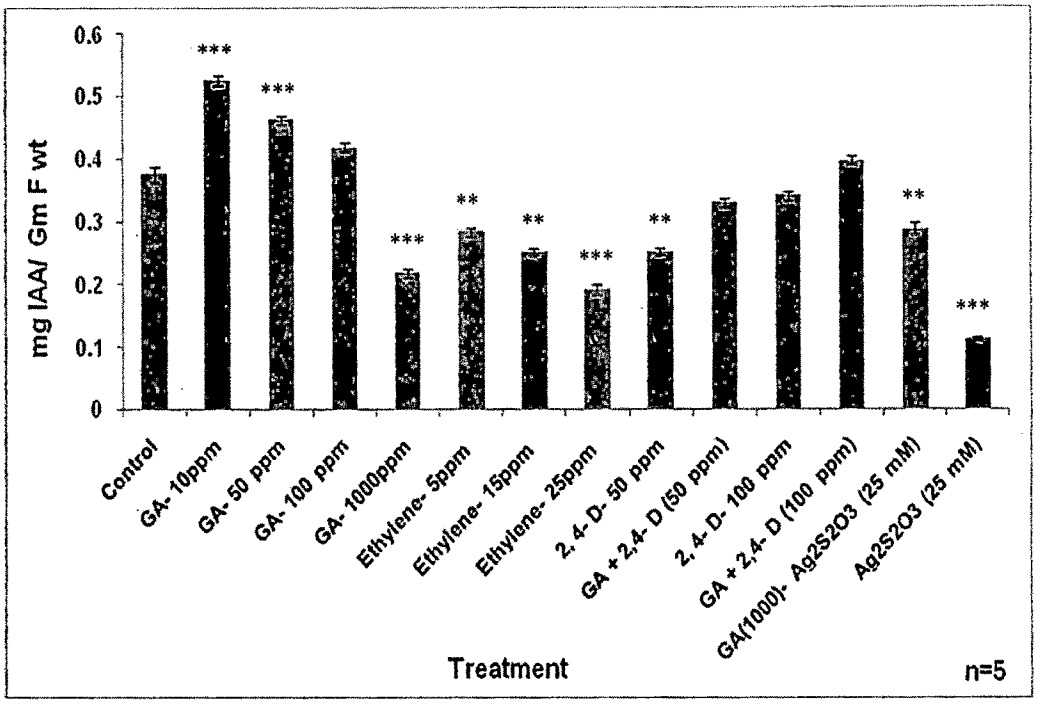

Figure 6.2: Effect of phytohormones on endogenous IAA level in floral bud

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

6.2.3. Estimation of Ethylene

The estimation of ethylene, a gaseous plant hormone is not easy. Ethylene gas can be easily prepared by decomposing ethephon in a buffered solution, and the resulting ethylene gas can be collected after incubation and used for analysis without any purification. Upon mixing ethephon with phosphate buffer, ethylene release is rapid and continuous for 24 hours. It slows down dramatically by day 3. In contrast, when dissolved in water, ethylene production was not detectable, possibly due to the acidity of the ethephon solution (Zhang and Wen, 2010). We attempted to simplify the protocol for ethylene estimation and the estimation of a key enzyme of ethylene synthesis. ACC, which is a key intermediate of Ethylene biosynthetic pathway.

Table 6.1: Standardization of Ethylene analysis with different concentration of Ethephon by Gas Chromatography

No.	Std. Ethephon (mM)	Time Incubation (Hours)	Area μ volt seconds	Retention time (seconds)
1.	10 mM	2.5	19.43	0.31
2.	10 mM	2	235.09	0.32
3.	10 mM	1.5	55.49	0.33
4.	10 mM	1.0	33.54	0.31
5.	50 mM	2.5	288.11	0.33
6.	50 mM	2	864	0.34
7.	50 mM	1.5	124	0.33
8.	50 mM	1.0	237	0.34
9.	1 mM	2.5	10,216	0.32
10.	**1 mM**	**2**	**40,011**	**0.29**
11.	1 mM	1.5	3397	0.33
12.	1 mM	1.0	2712	0.34

As shown in the Table 6.1 two hours is the optimum time for incubation for maximum ethylene formation. 1mM Ethephon concentrateion is sufficient for maximum evolution of ethylene and its detection.

6.2.4. Effect of Phytohormones on endogenous Ethylene level in floral bud during flower development

Using the protocol developed Ethylene production was monitored by Gas Chromatography in incubation vials based on the method of Lizada and Yang (1979) as mentioned in Methods and Material. Ethylene concentration was significantly increased with increase in levels of exposure to GA and Ethylene treatments (Figure. 6.3). Silver thiosulfate treatment shows significant minimum release of endogenous Ethylene. The

level of Ethylene significantly decreases in $Ag_2S_2O_3$ (25mM) treatment but Ethylene level increases in combination with GA treatment. In 2, 4-D (100 ppm) treated plants, Ethylene levels are below detection. There is a significant and measurable decrease in Ethylene levels in 2, 4-D (50 ppm) treatment compared with control. GA+2, 4-D (100 ppm) treated plant shows significant increase in Ethylene level compared to 2, 4-D alone.

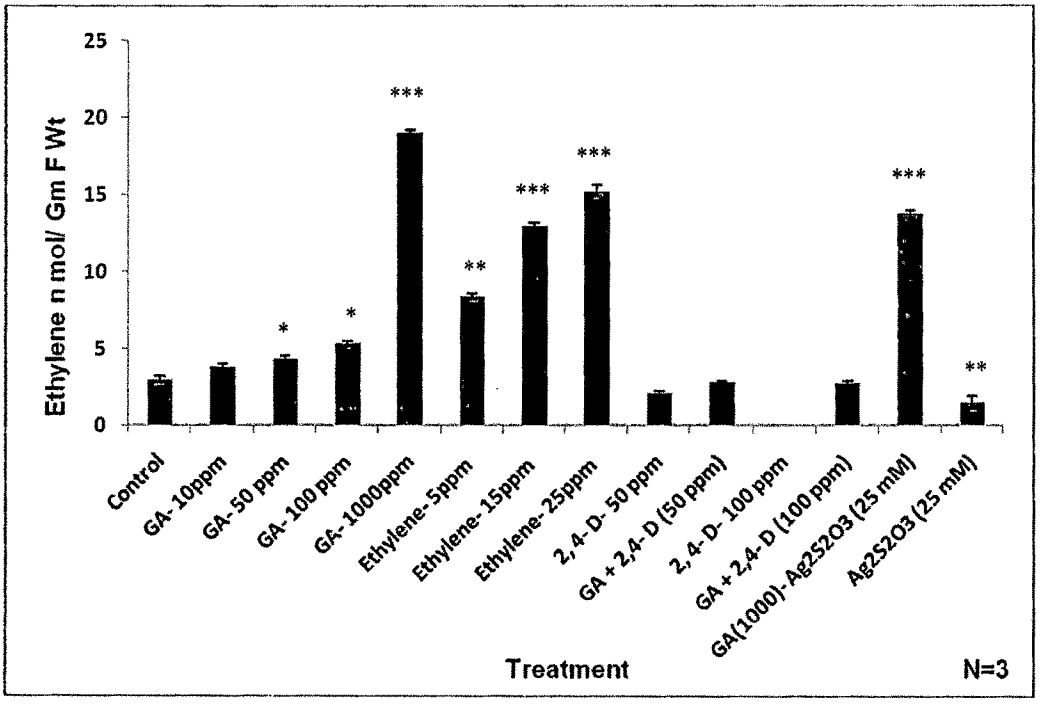

Figure 6.3: Effect of phytohormones on endogenous Ethylene level in floral bud

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

6.2.5. Effect of Phytohormones on ACC level in floral bud during flower development

ACC levels were monitored by Gas Chromatography by measuring the efficiency of the conversion of ACC to ethylene in incubation vials. ACC was extracted and its conversion to Ethylene was determined by Lizada and Yang (1979) method as

mentioned in Methods and Material. ACC levels were increased significantly with increase in levels of exposure to GA and Ethylene treatments (Figure 6.4). There is a significant decrease in the levels of ACC on 2, 4-D (100 ppm) treatment. GA+2, 4-D (50 and 100 ppm) treated plant shows significant increase in ACC level compared to 2, 4-D (50 and 100 ppm) treatment which suggests that GA increase the level of ACC which were inhibited by 2, 4-D. Silver thiosulfate treatment shows maximum level of ACC. The level of ACC significantly increases in plants treated with GA (1000)-$Ag_2S_2O_3$ (25mM) and $Ag_2S_2O_3$ (25mM) alone.

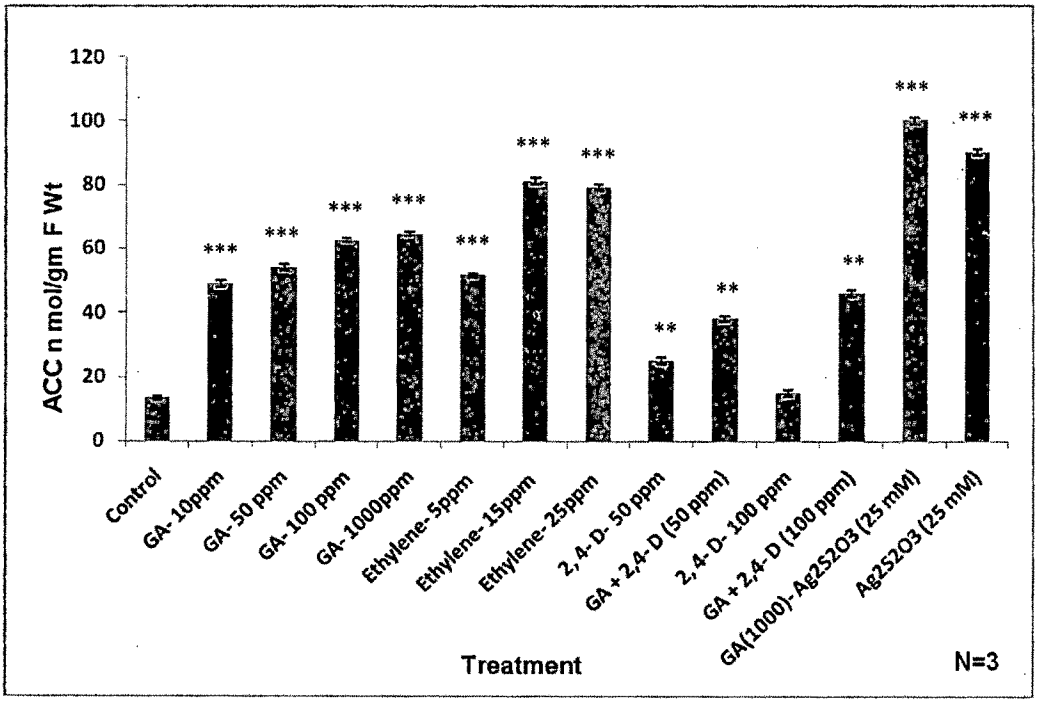

Figure 6.4: Effect of phytohormones on ACC level in floral bud

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

6.3. Discussion

6.3.1. Effect of phytohormones on endogenous GA level

GA and Ethylene treatment resulted in an increase in endogenous GA levels in a dose dependent manner. In contrast to this observation, Ethylene has been shown to represses the GA biosynthesis via DELLA stabilization in Arabidopsis (Archard et al., 2003). GA induced the transition of flowering in Arabidopsis under short days, and this effect was suppressed by Ethylene treatment at higher level (Archard et al., 2007). Thus, it could be concluded that, 25 ppm ethephon treatment may not be sufficient concentration to represses GA biosynthesis significantly. Silver thiosulfate treatment decreases the level of endogenous GA while Silver thiosulfate with GA increases endogenous level of GA. It suggests that inhibition of Ethylene not only delays flowering and development but also GA biosynthesis. This proves that activation of ethylene responsive genes is necessary for induction of flowering, which is also reported in Guzmania lingulata 'Anita' and Cucumber (Danijela Dukovski, 2004; Yamasaki et al., 2000). GA protects the deleterious effect of Silver thiosulfate and restores the flowering mechanism by releasing endogenous GA. Increased GA regulates development of flowers by activation of *LFY* and *API* genes (Jack, 2004). 2, 4-D with GA treatment promotes GA responses via destabilization of DELLA compared to 2, 4-D alone (Weiss and Ori, 2007). Therefore, Auxin could positively interact with GA either at the biosynthesis level or by promoting DELLA degradation.

6.3.2. Effect of phytohormones on endogenous IAA level

GA and Ethylene treatment shows decrease endogenous IAA levels in a dose dependent manner. Ethylene acts antagonistically to Auxin. Increased GA and Ethrel treatment negatively interacts with Auxin either at the biosynthesis level or at receptor level. It suggests that, as the GA and Ethrel treatment increases it activates Auxin oxidase which decreases the endogenous Auxin level (Wolbang and Ross, 2001; O'Neill and Ross, 2002; Frigerio et al., 2006) in pea (Ozga et al., 2003) and tomato (Serrani et

al., 2008). Silver thiosulfate with GA increases endogenous level of IAA compared to Silver thiosulfate alone. It suggests that, at specific dose there is a direct crosstalk between Auxin and ACS, which is mediated by Ethylene (Yi et al., 1999). It can also be interpreted that GA restores the harmful effect of Silver thiosulfate by release of endogenous IAA. GA with 2, 4-D treatment increases endogenous IAA compared to 2, 4-D alone. Hence, GA treatment with 2, 4-D promotes IAA through the regulation of DELLA protein resulting in the activation of the transcription factor *AUXIN RESPONSE FACTOR7 (ARF7).*

6.3.3. Ethylene Estimation

The decomposition of ethephon and the molar ratio of ethephon to ethylene is 1:1(Biddle et al., 1976). Ideally, 1 mol ethephon will yield 1 mol ethylene gas. According to the Ideal Gas Law, 1 mol of ideal gas has a volume of 24 L at 1 atmosphere and 20°C; 100 µmol ethylene gas will be 2.4 x 10^{-3} L. The measured ethylene concentrations were very close to the expected values, suggesting that nearly complete ethephon decomposition occurred in the buffered solution.

6.3.4. Effect of phytohormones on ACC and Ethylene levels

An assay whereby the level of ACC in plant tissues and organs can be determined is essential to an understanding of ethylene regulated physiological processes. ACC content and Ethylene was increased by the exogenous application of GA and Ethephon in a dose dependent manner. GA activates Ethylene biosynthesis or inhibits the suppression of ethylene biosynthesis via DELLA destabilization. 2, 4-D shows negative regulation of ACC and Ethylene release, but 2, 4-D with GA treatment restore the negative effect of 2, 4-D and increases ACC and Ethylene. Silver thiosulfate acts as a positive regulator of ACC leading to an increase in the level of ACC, however this in contrast to expected result acts as a negative regulator for Ethylene release. Silver thiosulfate complex is an inhibitor of ethylene action by virtue of its dissociation in the plant tissues to release silver ions. These ions act efficiently as anti-ethylene agents (Veen and Van De Geijn,

1978). 2, 4-D also acts like Silver thiosulfate to inhibit the release of Ethylene but molecular mechanism is unclear.

There is a positive interaction between Ethylene and GA and a negative interaction between Ethylene and 2, 4-D. Auxin delays the abscission zone formation via Ethylene inhibition. There will be inducton of several Ethylene responsive genes by GA. This study also suggests that the positive interaction between ethylene and GA mediates the timing of decision to flower in reponse to changing environment conditions and hormonal regulation.