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ORIGINAL PAPER

GA application induces alteration in sex ratio and cell death in *Jatropha curcas*

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Abstract *Jatropha curcas* L. is a commercially important plant with biodiesel and medicinal potential. It is a monoecious plant with staminate and pistillate flowers in same inflorescence with number of staminate flowers being higher than pistillate ones resulting in very low fruit yield. Altering sex ratio to increase the number of female flowers would lead to better yield. Phytohormones are most important factors known to alter sex ratio in plants. The mechanism by which phytohormones alter sex ratio differs in different plant species. Among phytohormones, GA plays an important role in sex alteration. In this study, we report the effect of exogenous application of GA on sex modification in *J. curcas*. There was considerable increase in number of female flowers by application of GA. At lower concentrations (10 and 100 ppm), increase in number of female flowers and fruit yield was proportionate to the concentration of hormone used but at higher concentration (1,000 ppm) though there was an increase in number of female flowers, fruit yield decreased. This was due to an increased peduncle length and enhanced cell death as a consequence of endogenous release of hydrogen peroxide in response to increased GA, resulting in withering of fruits.

Keywords Gibberellin (GA) · ppm—parts per million · Flower sex ratio · Hydrogen peroxide · Fruit fall · Cell death

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Introduction

Jatropha curcas is a plant with tremendous untapped potential as a source of biodiesel. It is a monoecious shrub with staminate (male) and pistillate (female) flowers being present on same inflorescence. A typical inflorescence has more male flowers as compared to female flowers, average male to female flower ratio being 29:1 (Solomon Raju and Ezradanam 2002). A change in flower sex ratio towards a greater number of female flowers could result into greater seed yield and hence greater oil yield. This would improve *J. curcas* potential as a biofuel crop. Various factors regulate floral sex expression in plants (Irish and Nelson 1989; Dellaporta and Calderon-Urrea 1994; Stehlik et al. 2008). Among these factors, phytohormones play a very important role (Ghosh and Basu 1983; Durand and Durand 1991). Of various phytohormones, role of Auxin, Ethylene and Gibbrellin (GA) in regulation of flower sex expression has been widely studied and is documented in recent studies (Tanimoto 2007; Thomas 2008; Salman-Minkov et al. 2008). Effect of exogenous phytohormones on flower sex ratio depends on species of plant. GA has been shown to promote female flowers in maize and castor bean, on the contrary it increases masculine features in hemp, spinach and cucumber (Lazarte and Garrison 1980; Pharis and King 1985). In some plant species such as Arabidopsis and Tomato, GA deficiency leads to male sterility because of abnormal anther development (Nester and Zeevaart 1988; Goto and Pharis 1999).

Though most studies have reported an increase in fruit yield in response to GA, Almeida et al. (2004), have reported decreased fruit yield in response to GA in oranges. They have attributed this to increased fruit withering as a result of endogenous release of hydrogen peroxide (H_2O_2) at the peduncle. GA has been shown to be responsible for

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inducing cell death by enhancing the ethylene biosynthetic pathway (Steffens and Sauter 2005). Programmed Cell Death (PCD) is a genetically defined process and is an integral part of plant ontogenesis. It is associated with morphological and biochemical changes (Jong et al. 2002). PCD plays key role in embryo development, formation and maturation of many cell types and tissues, and plant reaction/adaptation to the environmental conditions. It also occurs during terminal differentiation, formation of vessel members and tracheary elements, lysigenous aerenchyma formation, and aleurone degradation (Alan 2001; Gunawardena 2008).

Here, we report the role of GA in inducing sex alteration and cell death in *J. curcas*. GA at lower concentration alters sex ratio of flowers in favor of female flowers, resulting in high fruit yield. However, at higher concentration, increase in number of female flowers did not translate into fruit yield due to H_2O_2 mediated cell death.

Materials and methods

Experimental plants

Fifteen months old *J curcas* plants of Vishwamitri Railway Colony plantation, Vadodara, India, were selected for experiments. Gibberellic acid (GA) at three different concentrations 10 ppm, 100 ppm and 1,000 ppm were used. Solutions were prepared by dissolving GA in small volume of isopropyl alcohol and final volume was made up with demineralized water after adjusting pH to 7.5–7.8. A few drops of surfactant were added to the solution. Plants sprayed with demineralized water containing only surfactant were considered as control. The selected time of spray was early morning hours and spraying was initiated from the time foliar bud emerged. Each inflorescence received three sprays of equal volume of solution at an interval of 5 days. Ten plants were taken per treatment. Total number of flowers and sex ratio were calculated 1 week after the last spray whereas fruit yield was measured 1 month after the last spray. Test and control plants were tagged with appropriate labels to follow flower development till about one and half months. Fruit yield was observed for 3 months at an interval of 1 month. After each treatment, dried seeds were collected and weighed (Results reported are average of 20 seeds of each group).

Determination of H_2O_2

H_2O_2 extraction and estimation from plant tissues was carried out as described by Zhou et al. (2006). Fresh leaves (0.5 gm) were frozen in liquid nitrogen and ground to powder in a mortar with pestle, along with 5 ml of 5% TCA and 0.15 gm activated charcoal. This mixture was then centrifuged at 10,000 g for 20 min at 4°C. The supernatant was recovered and its pH was adjusted to 8.4 with 17 M ammonia solution before subjecting to filtration. The resultant filtrate was used to determine H_2O_2 levels. The reaction mixture containing 1 ml filtrate and 1 ml colorimetric reagent was incubated for 10 min at 30°C. In a similar way, 1 ml of distilled water was used as a blank instead of filtrate. Absorbance at 505 nm was determined spectrophotometrically.

Cell death study

Thin section of stem (peduncle part) was stained with Evans blue stain and observed under 40× objective of the light microscope. Evans blue stained only dead cells which appeared blue in color while live cells reduced the stain to a colorless form.

Statistical analysis

All the values are reported as mean ± S.E.M. The statistical significance was obtained by using Student's t-test.

Results and discussion

GA treatment, in a concentration dependent manner, resulted in an increase in total number of flowers and

Table 1 Effect of GA on flower sex ratio in *Jatropha curcas*

GA treatment	Number of plants treated	Number of inflorescences observed	Total flowers in inflorescences	Total male flowers	Total female flowers	Ratio female: male flower
Control	10	50	4220.9 ± 0.378	4040 ± 8.432	172.1 ± 0.481	1:24
10 ppm	10	50	5023.7 ± 0.471***	4791 ± 91.62***	232.7 ± 0.472***	1:21
100 ppm	10	50	6234.2 ± 0.489***	5846 ± 19.65***	388.9 ± 0.526***	1:15
1,000 ppm	10	50	6683.9 ± 0.481***	6193 ± 33.90***	490.1 ± 0.566***	1:13

Equal volume of GA was sprayed at three different concentrations 10, 100 and 1,000 ppm. Three such sprays were given at an interval of 5 days each. Numbers of flowers are calculated after the end of third spray. Values are mean ± SE, *** indicates significantly different at $P < 0.001$ as compared to the corresponding control

Table 2 Effect of GA on time scale of inflorescence development in *Jatropha curcas*

GA treatment	Duration (in days) for inflorescence development					
	Initial appearance of floral bud	Distinct appearance of flower bud	Distinct male and female flower bud	Opening of male flowers	Opening of female flowers	Complete bloom
Control	9.00 ± 0.577	6.333 ± 0.333	4.667 ± 0.333	6.160 ± 0.333	9.467 ± 0.333	36.20 ± 1.068
10 ppm	5.60 ± 0.509***	5.80 ± 0.583***	3.40 ± 0.748***	6.40 ± 0.748***	6.60 ± 0.812***	27.80 ± 0.812***
100 ppm	5.00 ± 0.447***	4.80 ± 0.374***	3.40 ± 0.509***	6.10 ± 0.871***	5.60 ± 1.02***	24.90 ± 1.02***
1,000 ppm	4.20 ± 0.374***	3.60 ± 0.374***	3.20 ± 0.583***	5.60 ± 1.020***	5.20 ± 1.249***	21.80 ± 1.249***

Emergence of floral bud was considered as day zero, Values are mean ± SE, *** indicates significantly different at $P < 0.001$ as compared to the corresponding control

Table 3 Effect of GA on peduncle length in *Jatropha curcas*

Treatment	Female flower (cm)	Male flower (cm)
Control	0.47 ± 0.18	0.55 ± 0.08
10 ppm	1.25 ± 0.14*	0.75 ± 0.14
100 ppm	3.25 ± 0.14***	1.16 ± 0.08*
1,000 ppm	3.90 ± 0.20***	1.41 ± 0.22*

Length of peduncle in flower starts to increase immediately after first spray. The length of peduncle was measured after the end of third spray. Values reported are mean ± SE; *, *** indicates significantly different at $P < 0.05$ and $P < 0.001$, respectively as compared to control ($n = 20$)

increased ratio of female: male flowers in all groups, highest effect being observed in plants treated with GA at 1,000 ppm (Table 1). The total number of flowers increased by 58% on 1,000 ppm GA treatment. Number of female flowers increased by almost three fold (Table 1) and this reflected in an appreciable and desirable increase in ratio of female: male flowers from 1:24 to 1:13. The role of GA in regulation of flowering has been well studied and molecular events involved have been deciphered. GA regulates the development of flowers by activation of LFY and AP1 genes. GA activates floral meristem LFY signal which up regulates AP1 promoter, responsible for flowering (Jack 2004). Thus, from this study it can be concluded that GA can be used as a potent phytohormone to increase flowering and seed yield from Jatropha plants.

Apart from increasing total number of flowers and female: male ratio, GA also hastened the process of flower development. Application of GA at 1,000 ppm, decreased

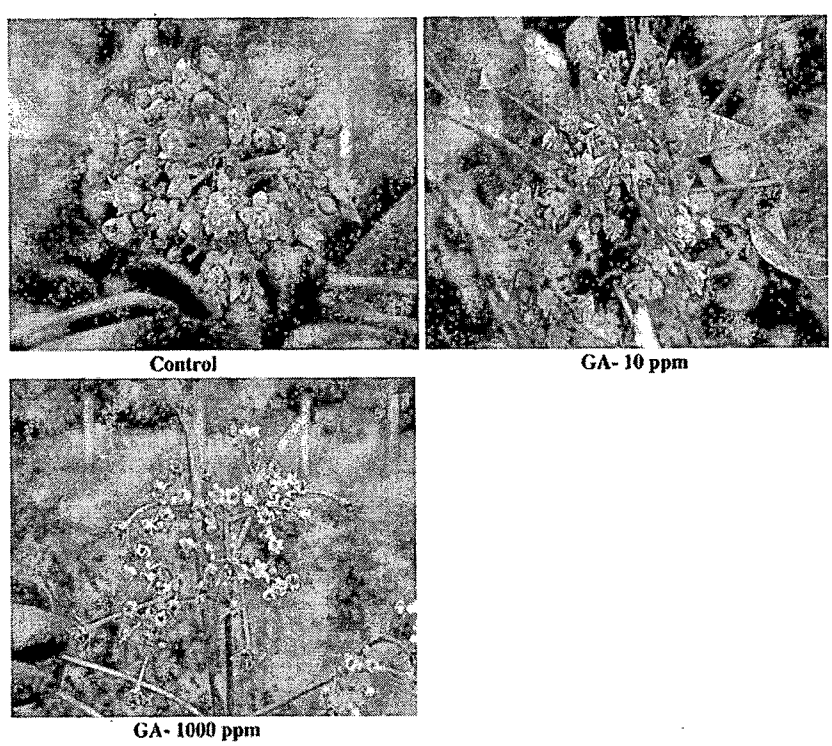

Fig. 1 Effect of GA on peduncle length in *Jatropha curcas*

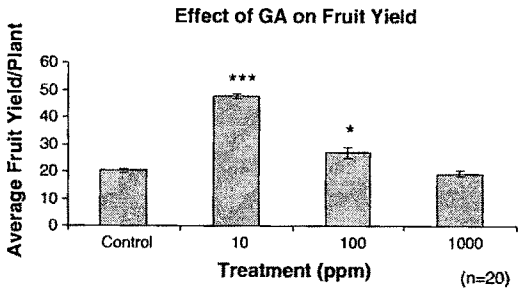

Fig. 2 Effect of GA on Fruit yield in *Jatropha curcas*. Numbers of fruits are calculated 1 month after third GA spray. Values represented is the mean of 20 replicates and bars indicate SE. *, *** indicates significantly different at $P < 0.05$, and $P < 0.001$, respectively as compared to control ($n = 20$)

Table 4 Effect of GA on endogenous release of Hydrogen peroxide in *Jatropha curcas*

Treatment	Hydrogen peroxide (μM/gm)
Control	8.738 ± 0.3287
10 ppm	10.61 ± 0.5916
100 ppm	13.86 ± 0.7699*
1,000 ppm	77.87 ± 1.267***

Hydrogen peroxide is determined by Zhou et al. (2006). Values are mean ± SE; *, *** indicates significantly different at $P < 0.05$ and $P < 0.001$, respectively as compared to the corresponding control ($n = 4$)

duration of flower development from 36 to 21 days as shown in Table 2. In GA treated plants, initial three stages of flower development were faster when compared to control. Time required for the entire process of flower development was shortened by nearly 12 days. Thus, GA not only increased flowering but also enhanced the rate of flower development.

Other obvious morphological change brought about by GA application was a pronounced increase in length of peduncle. In plants treated with 100 and 1,000 ppm GA, the length of peduncle of female flowers increased by 7 and 8 folds, respectively (Table 3); whereas the increase in peduncle length of male flowers was only 2 and 3 fold. This showed that GA effect on peduncle length was more prominent in female flowers compared to male flowers. GA at a lower concentration (10 ppm) showed negligible effect on peduncle length (Fig. 1). However, increase in length of peduncle was accompanied by greater withering of immature fruits. It was observed that there was higher fruit fall in plants treated with 100 and 1,000 ppm of GA as compared to 10 ppm. As a consequence when fruit yield was calculated, the highest yield was from plants treated with 10 ppm GA (Fig. 2). It has been reported that fruit fall is a consequence of senescence and programmed cell death and one of the important initiators of senescence is endogenous release of H_2O_2 (Strother 1988). There have been several reports which have demonstrated that GA treatment leads to release of endogenous hydrogen peroxide, a factor responsible for cell death (Bethke et al. 1999; Steffens and Sauter 2005). Bethke et al. (1999) have demonstrated the role of GA in accelerating ethylene induced cell death through generation of H_2O_2. However, the common signaling pathway between GA and ethylene leading to cell death has not been elucidated. Jong et al. (2002) have proposed that ethylene acts as a cofactor for H_2O_2 production by affecting phosphorylating signals which are involved in NADPH oxidase activation and catalase inhibition. Based on this premise, we estimated the levels of endogenous H_2O_2 to correlate PCD and fruit withering (Table 4). Plants treated with 1,000 ppm GA shows 10 times higher endogenous release of H_2O_2 compared to control, while no appreciable change was seen in plants treated with 10 ppm GA. Increased level of H_2O_2 is responsible for metabolic release of reactive oxygen species which leads to senescence resulting in cell death. Evans blue staining of sections from plants treated with 1,000 ppm GA showed an approximate 50% cell death (Fig. 3). H_2O_2 has been implicated in cell death by acting as a diffusible factor responsible for initiation of programmed cell death (Jong et al. 2002).

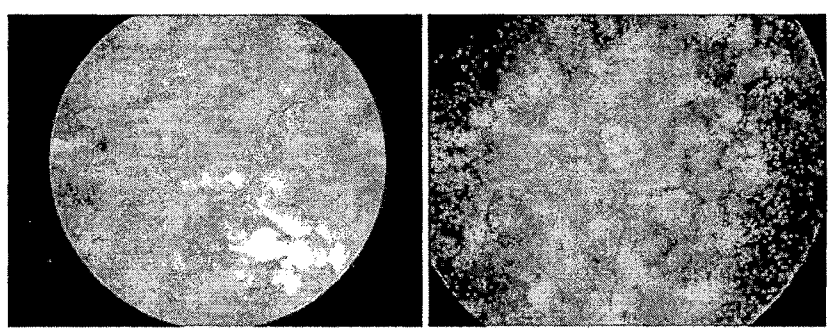

Fig. 3 Effect of GA on cell death in *Jatropha curcas*. Thin section of peduncle was stained with Evans blue stain and observed under 40× objective of light microscope. Evans blue stains only the dead cell which appears blue in color. In the living cells the dye is reduced to a colorless form

In summary, GA treatment of *Jatropha curcas* foliar bud increases the number of female flowers and also hastens the flower development. Higher number of female flowers at 1,000 ppm of GA did not translate into high fruit yield due to increased withering of immature fruits. Withering of fruits could presumably be due to cell death mediated by H_2O_2 release at the zone of abscission and greater peduncle length. If inhibiting release of H_2O_2 is realized, it would translate to an increase in female flowers and hence fruit yield. It would be a huge leap in per hectare production of *Jatropha* ensuring its commercial success. *Jatropha curcas* then can truly be an apt source of biodiesel.

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Comparing potential of GA and 2, 4-D in increasing fruit yield from Jatropha curcas

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ABSTRACT

Jatropha curcas L. has been accepted world wide as a plant with tremendous commercial importance as a source for biodiesel. It is a monoecious plant with staminate and pistillate flowers on same inflorescence. The number of staminate flowers is higher than pistillate ones resulting in very low fruit yield. Altering the sex ratio, to increase number of female flowers would lead to better yield. Phytohormones are one of the most important factors known to alter sex ratio in plants. The mechanism by which phytohormones alter sex ratio differs in different plant species. Earlier studies from our laboratory have shown effect of exogenous application of GA and 2, 4-D on flowering patterns in Jatropha. Here we report a comparative study of the effect of exogenous application of GA and 2, 4-D (50 and 100 ppm) on flower sex modification in Jatropha curcas. There was considerable increase in total number of flowers and female: male flower ratio by application of GA and 2, 4-D. However, at higher concentration of GA a negative result as far as fruit yield is considered was seen. The reasons for this are reported here. 2, 4-D on the other hand increased the ratio of female flowers in a concentration dependent fashion. Of the four treatments reported here, we have observed that 2, 4-D (100 ppm) shows better results than GA and untreated plants. The reasons for this are discussed.

Keywords: Gibberellin (GA), 2, 4-Dichlorophenoxyacetic acid (2, 4-D), ppm - parts per million, Flower sex ratio.

1. INTRODUCTION

India is one of the highest petroleum consuming nations in the world. India's economy has often been unsettled by its need to import petroleum. This coupled with deleterious effects of increased use of fossil fuels on the environment makes it imperative for us to find an environment friendly replacement. Bio-fuels, has been considered as a viable option to replace fully or partially India's dependence on fossil fuels. *Jatropha curcas* is a plant with tremendous untapped potential as a source of biodiesel. It is a monoecious shrub with staminate (male) and pistillate (female) flowers being present on same inflorescence. The inflorescence has more male flowers as compared to female flowers; the average male to female flower ratio being 29:1 [1]. This ratio leads to a very poor seed yield. A change in flower sex ratio towards a greater number of female flowers could increase seed yield and hence oil yield making the plant a commercially viable option. Phytohormones are important factors regulating floral sex expression in plants [2, 3, 4, and 5]. Among phytophormones the role of Auxin, Ethylene and Gibberellin (GA) has been widely studied and is documented in recent studies [6, 7, and 8]. The effect of exogenous phytohormones on flower sex ratio depends on species of plant. GA has been shown to promote female flowers in maize and castor bean, but on contrary it increases masculine features in hemp, spinach and cucumber [9, 10]. In several plant species such as Arabidopsis and Tomato, GA deficiency leads to male sterility because of abnormal anther development [11, 12]. Though most studies have reported an increase in fruit yield in response to GA, Almeida et al. [13], have reported decreased fruit yield in response to GA in oranges.

They have attributed this to increase immature fruit fall as a result of increased length of peduncle. Here, we report the role of GA and 2, 4-D in altering the number of flowers, female: male flower ratio, fruit yield and oil yield. The results reported here would have a significant commercial implication for biodiesel production.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL PLANTS

Young *Jatropha curcas* plants (15 months old) were selected for experiments. Gibberellic acid (GA) and 2, 4-D (2, 4- Dichlorophenoxyacetic acid) at two different concentrations of 50 ppm and 100 ppm were used. Solutions were prepared by dissolving phytohormone in small volume of isopropyl alcohol and final volume made up with demineralized water after adjusting the pH to 7.5-7.8. A few drops of Tween-20 were added to solution as surfactant. Plants were first sprayed at stage of foliar bud emergence. Plants sprayed with demineralized water containing only the surfactant were treated as control. The selected time of spray was early morning hours. Equal numbers of sprays per inflorescence were given for three times keeping an interval of five days between each spray. Approximately 5-10 ml of solution was used per inflorescence. Five plants were taken per treatment. Total flower sex ratio and flower number was calculated one week after last spray whereas fruit yield was calculated one month after last spray. Peduncle lengths were measured after first spray till fruit developed. Fruit yield were observed three times at interval of one month. Seeds were dried to constant weight. Twenty seeds were pooled together in each group for seed weight determination.

2.2. OIL AND FATTY ACID ANALYSIS

Dry mature seeds were analyzed for oil content after removal of seed coat using the method of Bligh and Dyer, [15]. Fatty acid profile of oil was determined by Gas Chromatography equipped with SS packed column and flame ionization detector.

2.3. STATISTICAL ANALYSIS

The data obtained was subjected to student's t-test. All values reported are mean $\pm$ S.E.

3. RESULTS AND DISCUSSION

Fifteen months old plants were used for this study. The foliar buds of the plants were sprayed thrice at an interval of 5 days with GA and 2, 4-D at two different concentrations (50 and 100 ppm) alone. Observations were made till the fruit development was complete. GA and 2, 4-D treatment resulted in an increase in total number of flowers in a concentration dependent fashion. GA at 50 and 100 ppm resulted in an increase in total number of flowers by 15% and 42 % respectively (Table 1). There was a negligible increase in flower number with 2, 4-D at 50 ppm while plants treated with 2, 4-D 100 ppm showed best result with an increase of 52%. The role of GA in regulation of flowering has been well studied and molecular events involved have been deciphered. It has been shown that GA regulates the development of flowers by activation of LFY and AP1 genes. GA activates the floral meristem LFY signal which up regulates AP1 promoter which results in flowering [16]. Thus from this study, it can be concluded that GA and 2, 4-D can be used as a potent phytohormone to increase yields from Jatropha plants.

The other obvious morphological change brought about by GA application was a pronounced increase in length of peduncle (Figure 1). This effect was more in peduncle of female flowers which showed an increase of 4 and 6 folds in plants treated with 50 and 100 ppm GA as compared to control (Figure 2). The peduncle of male flower also increased in length but increase was only 1 to 2 folds respectively at 50 and 100 ppm

Table 1. Effect of GA and 2, 4-D on flower number and sex ratio in *Jatropha curcas*

Treatment (ppm)	Number of in florescences observed	Total flowers in inflores-cences	Total Male flowers	Total Female flowers	Ratio Female:Male flower	Percent increase in total flowering
Control	20	2050 ±14.23	1970 ± 13.68	80 ± 0.55	1:25	—
GA (50)	20	2358 ± 8.05***	2226 ± 7.48***	132 ± 0.57***	1:17	15 %
GA (100)	20	2919 ± 11.83***	2749 ± 11.12***	171 ± 0.71***	1:16	42 %
2, 4-D (50)	20	2136 ± 4.489***	2025 ± 4.33***	112± 0.21***	1:18	4 %
2, 4-D (100)	20	3116 ± 12.09***	2920 ± 11.24***	196 ± 0.85***	1:15	52 %

Equal volume of GA and 2, 4-D was sprayed at two different concentrations 50 and 100 ppm. Three such sprays were given at an interval of 5 days each. Numbers of flowers are calculated after the end of third spray. Five plants were used in each treatment. Values are mean ± SE, *** indicates significantly different at $p<0.001$ as compared to the corresponding control.

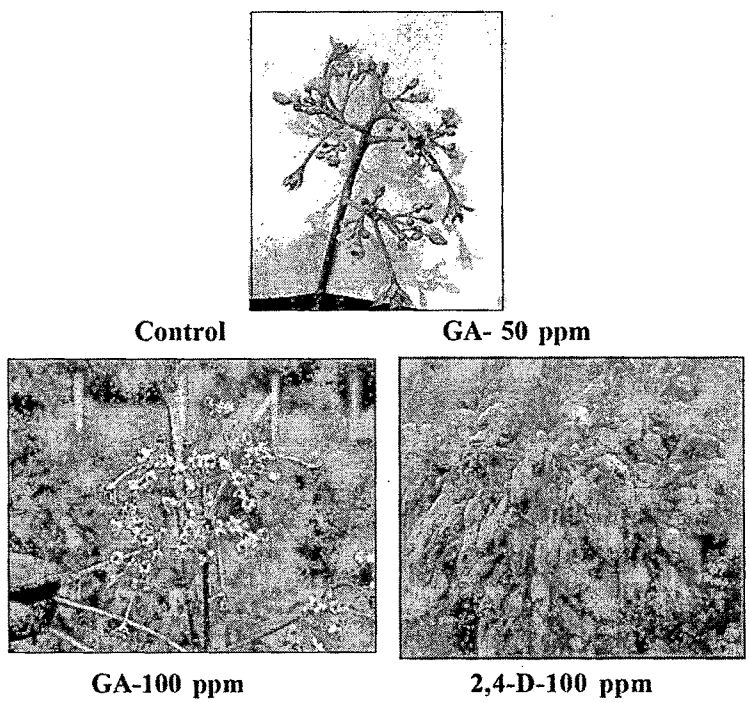
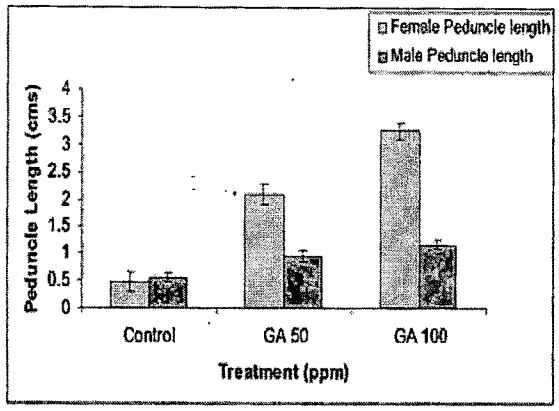


Figure 1. Effect of GA and 2, 4-D on peduncle length in *Jatropha curcas* The length of peduncle was measured after the end of third spray.



n=5

Figure 2. Effect of GA on peduncle length of *Jatropha curcas* Length of peduncle in flower starts to increase immediately after first spray. The length of peduncle was measured after the end of third spray. Values reported are mean ± SE; *, * indicates significantly different at p<0.05 and p<0.001 respectively as compared to control.**

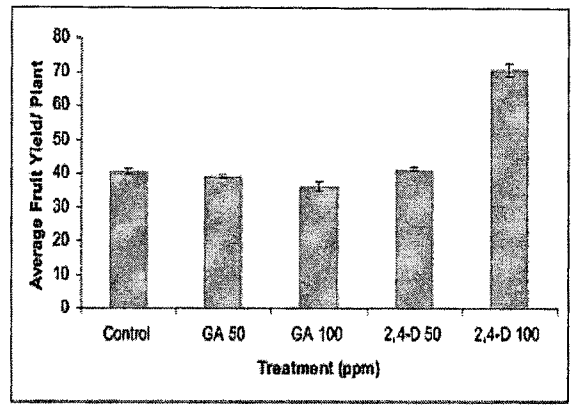


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Figure 3. Effect of GA and 2, 4-D on Fruit yield of *Jatropha curcas* Numbers of fruits are calculated one month after third GA and 2, 4-D spray. Values represented is the mean of 5 replicates and bars indicate SE, **, * indicates significantly different at p<0.01, and p<0.001 respectively as compared to control.**

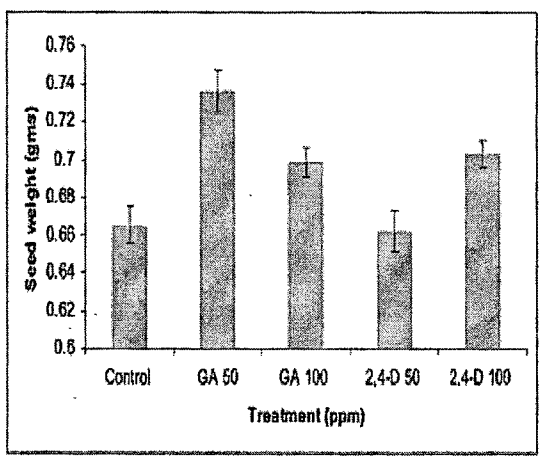
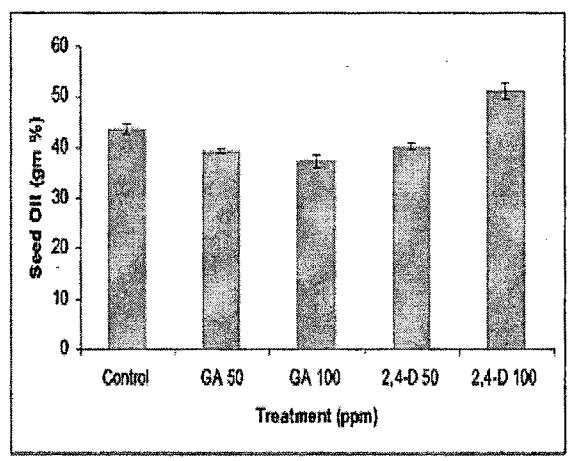


Figure 4. Effect of GA and 2, 4-D on Seed weight of *Jatropha curcas*

Dried seeds of each treatment were weighed (Results reported are average of 20 seeds of each group). Values are mean ± SE; *, ** indicates significantly different at p<0.05 and p<0.001 respectively as compared to the corresponding control.



n=5

Figure 5. Effect of GA and 2, 4-D application on Seed Oil yield of *Jatropha curcas* Dry mature seeds were analyzed for oil content by the method of *Bligh and Dyer* (1959). Values are mean ± SE; *, ** indicates significantly different at p<0.05 and p<0.001 respectively as compared to the corresponding control.

treatments of GA as compared to control. The flip side of an increase in length of peduncle was greater withering of fruits. It was observed that there was higher fruit fall in plants treated with 100 ppm of GA as compared to 50 ppm. Increased length of peduncle leads to weaker peduncle and it causes withering of fruits before it matures. Fruit fall is a process of senescence which is regulated by ethylene. However, there is a good crosstalk between GA and Ethylene which mediates the senescence in plant [17].

A consequence of increase peduncle length was fruit fall. When fruit yield was calculated the highest yield was from plants treated with 100 ppm 2, 4-D as compared to 50 and 100 ppm GA (Figure 3). 2, 4-D 100 ppm shows significant good results as compared to 2, 4-D 50 ppm. Also, there was no significant change in seed weight with GA and 2 4-D 100ppm treatments when compared with control. Seed weight was significantly higher in GA 50 ppm (Figure 4). The oil content in seeds showed appreciable decrease with GA treatment indicating that the fatty acid synthesis pathway is also not up regulated. The oil content in the seeds increases with 2, 4-D 100 ppm (Figure 5), which means that 2, 4-D 100 ppm increases the flux of fatty acid pathway. In conjunction with oil content, the fatty acid profile did not change by phytohormones application (Table 2).

Table 2. Effect of GA and 2, 4-D on Fatty Acids composition of *Jatropha curcas* seed oil

Fatty Acids composition	Control	GA (50 ppm)	GA (100 ppm)	2,4-D (50 ppm)	2,4-D (100 ppm)
Myristic acid	0.29	—	—	0.24	—
Palmitic acid	11.29	10.41	11.62	13.28	12.28
Palmitoleic acid	0.70	0.58	0.68	0.78	0.92
Stearic acid	4.56	3.89	4.23	4.80	4.51
Oleic acid	45.15	45.44	46.39	48.83	49.13
Linoleic acid	37.81	39.35	36.92	31.74	32.75
Linolenic acid	0.20	0.18	0.16	0.19	0.25

Fatty acid analyse from seed oil by Gas Chromatography. Values represents are g (%).

4. CONCLUSION

GA and 2, 4-D treatment on *Jatropha curcas* foliar bud increases the number of female flowers and fruit yield. GA at higher treatment increases female flowers however it did not result in correspondingly high fruit yield due to increased withering of immature fruits. Withering of immature fruits could be due to greater peduncle length. Using inhibitors to decrease peduncle length may help in capitalizing on the increase number of female flowers. 2, 4-D at higher concentration increases the number of flowers and fruit yield. Applying the results of such studies to the field could help to increase the potential of *Jatropha curcas* as a bio-fuel crop.

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