STRATEGIES TO IMPROVE SEED PRODUCTION IN *JATROPHA CURCAS* - A POTENTIAL SEED OIL CROP FOR BIODIESEL

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Abstract

Interest in planting *Jatropha curcas* L. for the production of biodiesel is growing exponentially. The properties of the crop and its oil have persuaded investors to consider *J. curcas* oil as a substitute for fossil fuels. However, this plant is still undomesticated, basic agronomic properties are not thoroughly understood and the environmental effects on growth have not been investigated. This thesis investigated different approaches that may contribute to improving the productivity of this plant.

Seed germination and methods of propagation are usually the first consideration in any plant development programme. The effects of aerosol smoke, smoke water, potassium nitrate, naphthalene acetic acid and indole-3-butyric acid on germination and seedling growth of *J. curcas* were investigated. Seed coat removal accelerated water imbibition and germination occurred within 48 h. Seeds exposed to aerosol smoke failed to germinate over the whole study period of three months. There were no significant differences in total germination between the treatments and the untreated control (intact- and shelled-seed). However, shelled-seeds had a shorter mean germination time. The seedlings were subsequently sown in trays under shade house conditions and different seedling growth traits measured after three months. Smoke water, potassium nitrate and naphthalene acetic acid produced significantly heavier seedlings with longer stems and roots, wider stems and a higher vigour index compared to the control treatments. Smoke water, potassium nitrate and naphthalene acetic acid stimulated seedling growth and vigour of *J. curcas*. This opens the possibility of applying these treatments to produce quality seedlings for large scale planting and accelerated plant establishment in production orchards.

Effective pollination is a prerequisite for many crops to increase seed-set and fruit production. Experiments were conducted to determine factors that could influence seed production in this potential biofuel seed crop. Controlled pollination experiments showed that plants required pollinator visits for seed production and were genetically self-

compatible. Pollen-supplementation did not lead to increased fruit set, suggesting that seed production in the study population was not pollen-limited. Both male and female flowers produced nectar and were highly attractive to honeybees. These insects were effective pollinators of *J. curcas*, as shown by experiments in which flowers exposed to single or multiple visits by honeybees set significantly more fruit than those from which visits were precluded. Pollinator-mediated self-pollination led to marginally lower levels of seed production relative to cross-pollination. Progeny from selfed plants had significantly shorter roots than progeny of outcrossed plants. However, in general, there was little evidence of inbreeding depression. The present results provide empirical evidence that honeybees are effective pollinators of *J. curcas*. Fruit arising from self-pollination were almost as numerous and as large as those arising from cross-pollination, suggesting that promotion of cross-pollination does not have to be a priority in orchard management for fruit yield.

Manipulation of pollen development and function is of vital importance for crop development and improvement. Experiments were conducted to investigate pollen viability, *in vitro* pollen germination and *in vivo* pollen tube growth in *J. curcas*. Light and fluorescence microscopy were employed to examine the different developmental stages. It was possible to determine pollen viability and distinguish between fresh and dead pollen using 2,3,5-triphenyltetrazolium chloride (TTC). Pollen germination was significantly higher in an agar-based medium composed of sucrose, boric acid and calcium nitrate compared with the control treatment (distilled water). Supplementation of IAA to the different media significantly increased pollen germination and pollen length compared with the control treatment. Pollen from hermaphrodite flowers had a lower viability, lower germination rates and shorter pollen tubes, with abnormal shapes, compared to the pollen from male flowers. Pollen tubes from both self- and cross-pollinated flowers entered the ovary within 8 hours after pollination (HAP). However, at 6 HAP, the pollen tube length and growth rate were significantly higher in cross- compared to self-pollinated pollen. Our results suggest that TTC is a reliable test for pollen viability; boric acid, calcium nitrate, sucrose and addition of IAA are essential and beneficial for pollen germination in this plant. Pollen germination and pollen tube growth

were not inhibited, nor interfered with, as a result of self-pollination treatments. During, both types of pollination, fertility is maintained as evidenced by ovule penetration by pollen tubes. This suggests that type of pollination has no influence on the success of fertilization in *J. curcas.*

Manual pruning is one of the major management practices in commercial plantations of *J. curcas*, resulting in production of more branches and thus increased potential for more inflorescences leading to a higher seed yield. Experiments were conducted to determine the response of *J. curcas* plants to manual pruning under summer and winter conditions. The results showed that manual pruning under both conditions significantly increased the number of branches per plant. However, there were no significant differences in number of branches between winter and summer manual pruning. Winter pruning, however, had a significantly wider crown diameter compared to the control and summer pruning. Both treatments produced significantly less fruits/per plant in the subsequent season compared to the un-pruned control. This study revealed that winter and summer manual pruning may be suitable practice to promote branching.

Manual pruning, however, is time consuming, labour intensive and expensive. A study was conducted to determine the potential of different plant growth regulators (PGRs) to increase the number of lateral branches of *J. curcas* plants. A single foliar application of BA (benzyladenine) at 12 mmol l^{-1} significantly increased branches in both the pot (4) and field (13.2) trials compared to manual pruning (MP) (1.8 and 5.7 respectively) and control (no new branches) plants. In the field, treatment with TIBA (2,3,5-triiodobenzoic acid) (1 mmol l^{-1}) significantly increased the number of branches (15.9) after seven months from application. Of all the PGRs examined, DK (Dikegulac) (2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid) at 2 mmol l^{-1} produced the maximum number of branches (18) in the field seven months after application. Concentrations of 2 and 3 mmol l^{-1} of MH (Maleic hydrazide) (1,2-dihydro-3,6-pyridazinedione, coline salt) significantly increased the number of branches, four and seven months after spraying in both the pot trial in the shade house and field respectively. Under field conditions *J. curcas* plants responded better to all the PGRs (DK < TIBA < BA < MH) when treated

once, with insignificant variations of other growth parameters. This study indicates that a single foliar application of PGRs under field conditions can be an alternative method to MP for increasing the number of lateral branches of *J. curcas* plants.

The field chemical pruning experiment was continued to determine the potential subsequent effects of the different PGRs on seed production. In the subsequent year following the single foliar application, the parameters of flowering, fruit set, fruit characteristics, total oil content and free fatty acid (FFA) content were evaluated. Number of flowers per plant and number of fruits per bunch were significantly affected by the different treatments. However, there were no variations in the degree of fruit set. A single foliar application of BA (6-benzylaminopurine) produced more flowers per plant, more fruits per bunch, heavier and bigger fruits and seeds with more oil compared to MP (manual pruning). TIBA (2,3,5-Triiodobenzoic acid) produced significantly more flowers per plant and heavier fruits compared to the control and MP treatments. However, it produced significantly bigger fruits with more seeds and a higher oil content than MP. DK (Dikegulac) (2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid) produced more flowers per plant and seeds with high oil content compared to the control and MP. However, it produced more fruit per bunch and more seeds per fruit compared to MP. MH (Maleic hydrazide) produced more flowers per plant, heavier and bigger fruits with numerous, heavier and oil rich seeds compared to the control and MP. This study indicates that foliar application of PGRs can be used in *J. curcas* to increase seed production and improve fruit quality.

STUDENT DECLARATION

STRATEGIES TO IMPROVE SEED PRODUCTION IN *JATROPHA CURCAS* - A POTENTIAL SEED OIL CROP FOR BIODIESEL

I, Hafiz Ahmed Abdelgadir, Student Number 205526858

declare that :

(i) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg;

(ii) This dissertation has not been submitted for any degrees or examination at any other University;

(iii) This thesis does not contain data, figures or writing, unless specifically acknowledged, copied from other researchers; and

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SIGNATURE

DECLARATION BY SUPERVISORS

We hereby declare that we acted as Supervisors for this PhD student:

Thesis Title: STRATEGIES TO IMPROVE SEED PRODUCTION IN *JATROPHA CURCAS* - A POTENTIAL SEED OIL CROP FOR BIODIESEL

Student's Full Name: Hafiz Ahmed Abdelgadir

Student Number: 205526858

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

SUPERVISOR: _____
PROFESSOR J VAN STADEN

CO-SUPERVISOR: _____
PROFESSOR S D JOHNSON

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List of abbreviations

2,4,5-T……..……..... 2,4,5-Trichlorophenoxpropionic acid

2,4-D……..……..... 2,4-Dichlorophenoxy acetic acid

4-CPA ……..……..4-Chlorophenoxyacetic acid

asl. ……..……...……..Above sea level

ACC……...1-Aminocyclopropane-1-carboxylic acid

Accel……..…… Tetrapyranylbenzyladenine

BA……..……..……..…… 6-Benzyladenine

CDM…….. Clean development mechanism

CKs ……..……..Cytokinins

COC ……..……Copper oxy chloride

DAP……..…….Days after pollination

DK……Dikegulac; 2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid

ER…….. Endoplasmic reticulum

FFA ……..……Free fatty acid

FCR……..……..…. Fluorochromatic reaction

FDA……..……... Fluorescein diacetate

GA……..……Gibberellin

HAP……..…….. Hour after pollination

IAA……...…….. Indole-3-acetic acid

IAAsp……..…… Indole-3-acetyl-L-aspartate

IBA ……..……Indole-3-butyric acid

IKI……...……...Iodine potassium iodide

MH……..……...Maleic hydrazide; 1,2-dihydro-3,6-pyridazinedione, coline salt

MMCs……..…… Microspore mother cells

MP……..…….. manual pruning

MTT……..…… ……2,5-Diphenyl tetrazolium bromide

NAA……..…… Naphthalene acetic acid

NAAm……...…… Naphthalene acetamide

NOx……...……..Nitrogen oxide

PGRs ……..……...Plant growth regulators

PM……Particulate matter

PMCs……..……...Pollen mother cells

RER……..…… Rough endoplasmic reticulum

SW……..Smoke water

TIBA……...……..……...2,3,5-Triiodobenzoic acid

TTC……..……..……2,3,5-Triphenyltetrazolium chloride

UV……Ultraviolet

δ.....Inbreeding depression value

L.....Pollen limitation index

1 Introduction

1.1 *JATROPHA CURCAS* – A POTENTIAL SEED OIL CROP FOR BIODIESEL

The rapid rise in crude oil prices and the geopotential uncertainty associated with ensuring uninterupted supplies have compelled researchers, economists and politicians to look for renewable substitutes. Liquid biofuels are widely recognized to be technically feasible alternatives. However, the jury is out to determine the environmental footprint of biofuels and the surrounding frenzy has often led to the announcement of unsustainable support prices for feedstock and nonviable procurement prices for the finished product (SRINIVASAN, 2009). Interest in using *Jatropha curcas* L. as a feedstock for the production of bio-diesel is rapidly growing. The properties of the crop and its oil have persuaded investors, policy makers and clean development mechanism (CDM) project developers to consider *J. curcas* as a substitute for fossil fuels to reduce greenhouse gas emissions. However, *J. curcas* is still an undomesticated plant in which many basic agronomic properties are not thoroughly understood and the environmental effects on cultivation have not been investigated yet (ACHTEN *et al.,* 2008).

Jatropha curcas is a multipurpose plant with many desirable attributes and considerable potential. It is a tropical plant that can be grown in low to high rainfall areas and can be used to reclaim land, as a hedge and/or as a commercial crop (OPENSHAW, 2000). *Jatropha curcas* is attractive for many reasons: it is a renewable energy source; balances CO_2 in environment; produce less harmful emissions than fossil fuel; the fuel production technology is simple; it is a non-edible oil source; is a perennial crop having a 30 year long life span; high oil content in seeds comparative to other biodiesel sources; is a disease-resistant plant; is not over-sensitive to climatic change; can be grown in arid areas; and due to its dormancy characteristics, it survives in various weather conditions (DANGE *et al*., 2006). However, there are also many problems with this plant: lack of good quality seeds and planting material; most of the information is

based on assumptions; non-availability of genuine and authentic data and information; no tried and tested cultivation experience; variable output; variable oil content; long gestation period of crop; no model available for effective use of by-products; and it currently has no economic viability as a mono-crop (DANGE *et al*., 2006). This is expected to change as fossil fuels become scarce and thus more expensive.

1.1.1 Common names, taxonomy and botanical description

There are many names for *J. curcas*, such as: Physic nut (HELLER, 1996; ACHTEN *et al.,* 2008; GRESSEL, 2008); Black vomit nut (MAKKAR *et al.,* 1998; GRESSEL, 2008); Purging nut or Purge nut (SIRISOMBOON, 2007; GRESSEL, 2008); Habb-EL-Meluk (MAKKAR *et al.,* 1998); Barbados purging nut (MAKKAR *et al.,* 1998).

Physic nut, *Jatropha curcas* L. (Euphorbiaceae), is a tropical plant native to Mexico and Central America (HELLER, 1996). The genus *Jatropha* L. is a morphologically diverse genus of 160 – 175 species of trees, shrubs, rhizomatous sub-shrubs or geophytes having a narrow geographic range in seasonally dry tropical regions (DEHGAN, 1984). *Jatropha curcas* was placed in *Jatropha* subgenus *Curcas* (Adans.) Pax, section. *Curcas* (Aans.) Griseb. Subgenus *Jatropha* includes all African (except two species), Indian (except one species), South American, Antillean, and two relict North American taxa. Subgenus Curcas included all of the Mexican, one Costa Rican, two African and one Indian species (DEHGAN, 1984). DEHGAN (1980, 1982); DEHGAN and CRAIG (1978); DEHGAN and WEBSTER (1979) considered *J. curcas* the most primitive member of the genus because it has palmately lobed leaves, an arborrescent growth habit, and occasional hermaphroditic flowers. Evolution was thought to have proceeded toward specialization in vegetative structures, culminating in a facultatively annual growth habit in section *Jatropha* in a rhizomatous-shrub habit concomitant with polyploidy (2n = 4x = 44) in section *Mozinna* (Subgenus *Curcas*). In subgenus. *Curcas* the inflorescence was drastically reduced to a few or solitary terminal or lateral flowers together with a gradual change from monoecy to dioecy. The evolution of flowers in subgenus *Jatropha* resulted in reduction and arrangement of stamens (from ten to eight, uni- or bi-seriate, monodelphus or free) without change in the number of locules of the fruit, while in

subgenus *Curcas* (except section *Curcas*) the number and arrangement of stamens remained unchanged, but the locules of the fruit and stigma lobes were progressively reduced from three to one (DEHGAN and WEBSTER 1979). These reductions and modifications coincided with South to North latitude and increasing aridity (DEHGAN, 1982).

Jatropha curcas is cultivated in many Latin American, Asian and African countries as a hedge. It is a small tree or large shrub, which can reach a height of up to 5 m (HELLER, 1996; GÜBITZ *et al*., 1999; MARTÍNEZ-HERRERA *et al*., 2006). The bark is smoothly gray and excudes a whitish coloured water latex when cut (SINGH *et al.,* 2006). Leaves are smooth, heart shaped, 4-6 lobed and 10-15 cm in length and width, initially light violet later on yellowish green and at maturity they become dark green, arranged alternately, and leaf fall occurs in the winter (GOUR, 2006). The plant is monecious and flowers are unisexual; occasionally hermaphrodite flowers occur (DEHGAN and WEBSTER, 1979). Plants flower during the wet season and two flowering peaks are often seen. However, in humid regions flowering occurs throughout the year (SINGH *et al.,* 2006). Inflorescences are formed terminally on branches and are complex, possessing main and co-inflorescences. Normally, the inflorescences produce a central female flower surrounded by a group of male flowers. In a few, the places where female flowers are expected are substituted by male flowers (RAJU and EZRADANAM, 2002). The average male to female flower ratio is 29: 1. In a previous study CHANG-WEI *et al*. (2007) reported that the male flowers opened first and a few flowers bloomed each day in each raceme. A large number of female flowers opened from day-3 to day-5 after the male flowers opened. Male flowers are small and salver-shaped. Sepals and petals are five each, free; the latter are connivent at the flower base, forming a short tube (RAJU *et al.,* 2002). There are five roots: one taproot and four lateral roots (HELLER, 1996). After pollination, the inflorescences form a bunch of green ellipsoidal fruits which produce grey-brown capsules, 4 cm long and generally tri-halved, each comprised of one seed. Seeds are black, about two cm long and one cm thick. The seeds mature three months after flowering (SINGH *et al.,* 2006).

1.1.2 Distribution

Jatropha curcas is native to Central America and Mexico where it occurs naturally in the forests of coastal regions. It is cultivated in Africa and Asia (BENGE, 2006). It is thought to have been distributed from the Caribbean by Portuguese seafarers via the Cape Verde Islands and Guinea Bissau to other countries in Africa and Asia (HELLER, 1996). However, *J. curcas* is almost pantropical now, and although toxic, it is widely planted as a medicinal plant. A non-toxic variety is reported to exist in Mexico and Central America (HENNING, 2000). It was reported that *J. mahafalensis* which is endemic to Madagascar has equal energetic promise. In many parts of Africa *J. curcas* is widely planted as a hedge or living fence to protect field crops since the foliage is toxic to animals (BENGE, 2006).

1.1.3 Ecology

Jatropha curcas is able to thrive in a number of climatic zones with rainfall 250–1200 mm. It is well adapted to arid and semi-arid conditions and has low soil fertility and moisture demands (KATWAL and SONI, 2003). *Jatropha curcas* is not self-propagating, it has to be planted (HENNING, 2000). The current distribution shows that introduction has been most successful in drier regions of the tropics with an average annual rainfall between 300 and 1000 mm. The plant occurs mainly at lower altitudes (0-500 m). It is not sensitive to day length. *Jatropha curcas* can withstand only a very light frost that causes it to lose all of its leaves, and the seed yield will probably sharply decline (BENGE, 2006).

1.1.3.1 Soils and soil fertility

Jatropha curcas grows best on well-drained soils with good aeration but is well adapted to marginal soils with low nutrient content; although in heavy soils root formation is reduced (BENGE, 2006). It can also grow on moderately sodic and saline, degraded and eroded soil (KATWAL and SONI, 2003). *Jatropha curcas* can grow in clay soils if

water logging or saturation occurs due to climatic conditions. In general heavy clay soil that swell and shrink (Montmorillonite) are not suitable because root system development is impaired. Sandy loamy soils seem to be best (OUWENS *et al*., 2007). Acid (PH < 6) or alkaline (pH > 8.0) soils are not suitable for *J. curcas*. *Jatropha curcas* can be well established on marginal soils and can reach reasonable production, if proper care is given to boost plant growth in the initial growth phases and it will maintain production with additional inputs (CHAUDHARRY *et al*., 2007; PATOLIA *et al*., 2007a; PATOLIA *et al*., 2007b, OGUNWOLE *et al*., 2007). As a perennial crop, *J. curcas* invests a decreasing fraction of its carbohydrates into the woody standing biomass over time. If properly pruned, the seasonal requirements for nutrients are only needed for the seasonal formation of branches, leaves, flowers, fruits and seeds. If senescent plant material, like leaves, flowers and pruned branches are left in the field or incorporated in the soil as mulch, they are slowly decomposed, resulting in the release of the nutrients back into the soil where they are available again for crop uptake. The toxic components (phorbol esters) of *J. curcas* decompose quickly as they are very sensitive to elevated temperatures, light and atmospheric oxygen (NIH, 2007).

1.1.3.2 Rainfall and humidity

Ranging from tropical very dry to moist through subtropical thorn to wet forest life zones, *J. curcas* grows well with more than 600 mm of rainfall per year and it withstands long drought periods. However, when rainfall is less than 600 mm it cannot grow except in special conditions like on Cape Verde Islands, where the rainfall is only 250 mm, but the humidity of the air is very high (rain harvesting) (BENGE, 2006). *Jatropha curcas* can survive precipitation as low as 300 mm/year by shedding its leaves, but it does not produce well under such conditions. The minimum rainfall to produce fruits is 600 mm/year and the optimal rainfall is 1000 – 1500 mm/year . Rainfall induces flowering as well as drought. A short period of drought might induce flowering. The cycle of flowering can thus be influenced using irrigation. High humidity or high rainfall can result in more fungal attacks to which the plant is sensitive (JONGSCHAAP *et al*., 2007).

1.1.3.3 Light and photoperiod

Jatropha curcas is not sensitive to day length (BENGE, 2006). Adult leaves of *J. curcas* are well adapted to high radiation intensities (BAUMGART, 2007).

1.1.3.4 Water use efficiency

To produce a mol of *J. curcas* oil, 57 mol CO_2 are needed in the photosynthesis process (JONGSCHAAP *et al*., 2007).

$$12\ H_2O + 6\ CO_2 + \text{light} \longrightarrow C_6H_{12}O_6 + 6\ O_2 = 6\ H_2O.$$

For the photochemical production process *J. curcas* oil needs 57 C (≈12 g mol^{-1}), 107 H (≈1 g mol^{-1}) and 6 O (≈16 g mol^{-1}). A mmol of *J. curcas* oil therefore, weight about 0.888 g. at a water use efficiency of about 3 mmol CO_2 per mmol H_2O, about 57/3 = 19 mmol H_2O (0.342 g H_2O) is needed to produce 0.888 g of oil. This is equivalent to 0.342/0.888 = 0.385 g water. g^{-1} oil, or 0.385 liter water kg^{-1} of oil, or 385 g water kg^{-1} oil, or is (at density of about 0.92 kg l^{-1}) equivalent to 0.345 liter water liter oil^{-1}. This value does not reflect the real water requirements and water use efficiency of *J. curcas*, as transpiration for plant cooling and other processes, such as transport functions, requires water as well (JONGSCHAAP *et al*., 2007).

1.1.4 General uses

All parts of *J. curcas* can be used for a wide range of purposes. Exploitation of *J. curcas* was described by GÜBITZ *et al*. (1999); OPENSHAW (2000); AUGUSTUS *et al*. (2002); and WOOD (2005).

1.1.4.1 The whole plant

The plant is widely cultivated in the tropics as a living fence in fields and settlements. This is mainly because it can easily be propagated by cuttings, can be densely planted for this purpose, the species is not browsed by cattle and it has a long life span (HELLER, 1996; SIRISOMBOON, 2007). Because of its drought tolerance and its lateral roots near the surface *J. curcas* is often used for anti-erosion measures, either in the form of a plantation together with other species, or in the form of hedges to reduce wind speed and protect small earth dams or stone walls against runoff water (HELLER, 1996).

1.1.4.2 The fruits and seeds

Fruit hulls have no significant value as fodder, so it is best to use them as mulch or compost. They can also be burnt in fuel-efficient cooking stoves. The seeds can be processed (oil, press cake) or sold directly as seed or for industrial use. The seeds contain 32 to 35 % oil (HELLER, 1996).

1.1.4.3 The pressed cake

With mechanic oil expellers, up to 75 - 80 % of the oil can be extracted. The press cake constitutes some 70 - 80 percent of the total mass of the seeds, depending on the extraction rate. The press cake cannot be used in animal feed because of its toxic properties. Because of its nitrogen (6 % N_2), phosphorous (2.75 % P_2O_5) and potassium (0.94 % K_2O) content, which is similar to that of chicken manure, it is valuable as organic manure. In practical terms, an application of 1 t of *J. curcas* press cake is equivalent to 200 kg of mineral fertiliser per hectare ("bulkblend" 12:24:12). Due to its residual oil content, the *J. curcas* press cake also has insecticidal properties, and reduces the number of nematodes in the soil (HELLER, 1996).

1.1.4.4 Medicinal uses

All parts of the plant, including seeds, leaves and bark, fresh or as a decoction, are used in traditional medicine and for veterinary purposes. The oil has a strong purgative action and is also widely used for skin diseases and to soothe pain such as that caused by rheumatism (1996). The sap (latex) has antimicrobial properties against *Staphylococcus* and *Streptococcus* spp. Latex from the stem is used to arrest bleeding of wounds (HELLER, 1996; SIRISOMBOON, 2007). Some of the medicinal uses according to HELLER 1996 are:

- A decoction of leaves is used against cough and as an antiseptic after birth;
- Branches are used as chewing sticks in Nigeria;
- The sap flowing from the stem is used to arrest bleeding of wounds. This is due to wound-healing properties of curcain, a proteolytic enzyme isolated from latex;
- Latex has antimicrobial properties against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Candida albicans*;
- It has coagulating effects on blood plasma;
- Extracts from physic nut fruits showed pregnancy-terminating effects in rats. However, there was uncertaininty whether the embryotoxic effect is due to a specific action or a result of general toxicity;
- A methanol extract of physic nut leaves afforded moderate protection for cultured human lymphoblastoid cells against the cytopathic effects of human immuno deficiency virus; and
- Extract of the leaves showed potent cardiovascular action in guinea pigs and might be a possible source of beta-blocker.

1.1.4.5 Plant protection and insecticides

Aqueous extracts from the leaves were effective in controlling *Sclerotium* sp., an *Azolla* fungal pathogen. Ground seeds showed molluscicidal activity against the host of liver fluke (*Lymnaea auricularia rubiginosa*), a disease which is widely distributed in the Philippines and also against the hosts of *Fasciola gigantea* and *Schistosomia* in Senegal. Extracts from crushed whole seeds showed molluscicidal activity against several schistosome vector snails. Phorbol esters are probably the active agents in the different extracts used (Heller, 1996).

1.1.4.6 Oil

The oil can be used in soap production giving a very good foaming. The white soap has a positive effect on the skin, partly due to the glycerin content. Research has been conducted into developing cookers that would run on plant oil, but no practical results have yet been achieved. Well-refined oil is a good basic material for cosmetics, but it is not yet used on a large scale. *Jatropha curcas* oil and press cake has been used to produce biocides (insecticide, molluscicide, fungicide and nematicide) (HELLER, 1996). The active components are phorbol esters, which gives the *J. curcas* press cake and oil its toxic property (HELLER, 1996). The oil can be directly used in older diesel engines or new big motors running at constant speed (pumps, generators). Blending with fossil diesel and/or other fossil fuels are other options. The oil can also be transesterified into *J. curcas* (m)ethyl esters that can be used in conventional diesel engines or diesel engines with adapted parameters (ACHTEN, 2008).

1.1.5 Cultivation and cultural practices

1.1.5.1 Propagation, sowing method and spacing

1.1.5.1.1 Propagation by seeds

Germination is fast and under favourable conditions it is completed in 10 days. Seeds are sown in the nursery beds in lines at an interval of 15 cm with a seed depth of 4 cm. Seed to seed distance in a line is kept at 5 cm. However, it was reported that the overnight soaking of seeds in water improves the germination percentage. Germination starts after 5-6 days and continues for 10-15 days. Dry seeds can also be sown but the germination processes are delayed (SINGH *et al.,* 2006).

1.1.5.1.2 Propagation by cuttings

Cuttings can be taken from one-year-old shoots. The best cuttings are taken from the middle of the branch.Thick strong shoots of 20-25 cm long with 4-5 buds are preferable as they give nearly 80-90% rooting. The cuttings are planted in raised beds 3-5 m long and 1.5 m wide. The soil is mixed with powdered and well rotten farm yard manure. The cuttings are planted closely with a spacing of 15-20 cm. The beds are watered regularly. For the quick establishment of hedges and plantations for erosion control, planting cuttings directly is recommended, whereas for long-lived plantations and vegetable oil production, plants propagated by seeds are better. The seeds germinate within a week and become ready for transplanting in 45 days. Plants grown from seed develop a typical tap root and four lateral roots (SINGH *et al.,* 2006).

1.1.5.2 Fertilization

Fertilization by organic or inorganic fertilizer increased seed yield by 100% (PATOLIA *et al.,* 2007a; PATOLIA *et al.*, 2007b). Fertilization experiments on marginal land in India showed that fertilization with nitrogen and phosphorus significantly increased plant

height, leaf area index (LAI), total above ground dry matter, seed yield and oil content in the second year (PATOLIA *et al.,* 2007a). Fertilization with *J. curcas* seed cake increased seed yield (GOSH, *et al.,* 2007). Inoculation with mycorrhiza significantly increased the uptake of phosphorous and microelements from ash (SHARMA, 2007b).

1.1.5.3 Irrigation

Irrigation is required for seedlings, especially during the first 2-3 months after planting. The requirement for water is contingent upon local soil and climatic conditions. During the dry period, life-saving irrigation may be given at time intervals depending on the requirement. Drip irrigation is not ideal as it induces too much vegetative growth. The critical stages of irrigation are: at transplanting, dry spells during summer in the first year of plantation for survival in rain-fed areas, and at flowering (to control sex switching and promote anther dehiscence). Frequency of irrigation needs to be calculated according to economics and water availability. Soon after planting, irrigation followed by laying of newspaper around plants have been found very effective for the initial establishment of saplings. Any material available as mulch will help to conserve moisture for establishment of saplings (GOUR, 2006).

1.1.5.4 Pruning

Crop architecture plays an important role in *J. curcas*. Proper pruning helps to produce more branches and healthy inflorescences to enhance good fruit set and ultimately improve yield. The pruning of terminals is essential in six-month-old plants to induce lateral branch formation. However, pruning at 30 cm height is ideal to manage. Likewise the secondary and tertiary branches are to be pruned at the end of the first year to induce a minimum of 25 branches and 35-40 branches at the end of the second year. Periodical pruning can be carried out depending upon the vegetative growth of the plants (GOUR, 2006). However, the pruning should be done when the tree sheds leaves and enters into a period of dormancy, preferably during the winter season. The trees are

kept short to better manage them during flowering and fruiting. This, also provides ease of movement during harvesting. Canopy management is advisable in trees with terminal bearing. Plant types with a branch in every leaf axil should not be pruned vigorously. The entire plant has to be cut to ground level leaving a 45 cm stump, once in 10 years. Re-growth is quick and yielding starts again in about a year (GOUR, 2006).

1.1.5.5 Harvesting

For oil purposes the seeds are harvested at maturity. The capsules are harvested when they turn yellow. The pods are collected manually and seeds are separated mechanically or manually. Seeds for planting purposes are dried in sheds, while for oil purposes they should be dried in the sun for four days (6-10% moisture level) before packing (GOUR, 2006).

1.1.5.6 Post-harvest processes

Seeds stored in ambient conditions can maintain their viability for 7-8 months. Longer storage affects seed viability. Therefore, seeds being used for plantation purposes need to be kept at low temperatures to protect them against loosing viability and for effective emergence. The oil industry requires a continuous supply of raw material for oil extraction and esterification. Seeds must be properly stored and prepared for extraction, to maintain a high quality in the final oil product. Long storage of seed is reported to affect oil quality and quantity hence long storage should be avoided. Drying of seeds to 4% moisture content enhances storage life (GOUR, 2006).

1.1.6 Pests and diseases

The occurrence of diseases and pests are highly region specific. *Jatropha curcas* has no serious pest or disease problem at present. However, this may change when it is grown in commercial plantations with regular irrigation and fertilization (GOUR, 2006). The toxic

characteristics of *J. curcas*, caused by constituents in leaves, stems, fruits and seeds may suppress damaging effects from some predators. However, in plantations especially under humid conditions, serious problems have been reported with fungi, viruses and attack by insects (SHARMA and SARRAF, 2007a).

1.1.6.1 Diseases

Collar rot may be a problem at the start of cultivation. This can be controlled with 0.2% copper oxy chloride (COC) or 1% Bordeaux drenching. Rot may become a serious problem in some areas during monoculture under irrigated conditions. It is caused by *Macrophomina phaseolina* or *Rhizoctonia bataticola.* Rotting at the adult stage has been observed in soils saturated with moisture for a long period of time. *Cercospora jatrophae-curcas* leaf spots are reported to be associated with this species. The rot can be controlled by application of 1 % Bordeaux drenching. Minor diseases such as root rot (*Fusarium moniliforme*), damping off (*Phytopthora* spp.) and leaf spots are reported to be caused by *Helminthosporium tetramera* and *Pestalotiopsis* sp. (GOUR, 2006).

1.1.6.2 Pests

According to GOUR (2006) the major pests affecting the plant are: Beetles, hoppers and leaf minor, blue bug, locust, green stink bug bark eater, capsule borer; and mites.

These pets can be controlled by:

- Mixtures of vitex, neem, aloe, Calatropis or Rogor at 2 ml l^{-1} of water;
- Endosulfan at 3 ml per litre of water; and
- Wettable sulfur against mite.

1.1.7 Yield and economics

Reliable yield predictions still forms a major problem as there are no reliable field data on dry *J. curcas* seed yield ha^{-1} yr^{-1} in a given set of conditions and at a certain level of input. In order to tackle this knowledge gap, it is necessary to systematically monitor the year-to-year seed yield in operational plantation conditions along with the influencing factors (ACHTIN, 2008).

According to OUWENS *et al*. (2007) yields of *J. curcas* range from extremely low to high. These variations may be explained by differences in the following growth and production related factors:

- *Age,* yields increase with age. It is therefore important to indicate at which age yields have been measured;
- *Soil conditions,* waterlogged soils and frost susceptible areas are not suitable for *J. curcas* which seems to be very sensitive to limited oxygen supply to roots;
- *Water availability,* differences in rainfall, length of the dry season and irrigation practices;
- *Nutrient availability*, due to different soil fertility levels;
- *Pests and diseases*, in different degrees of incidence and length, according to the ecological conditions; and
- *Genetic factors,* This is a common feature; a strict selection of seeds or cuttings leads to more uniformity in offspring and higher yields per plant.

However, ACHTIN (2008) highlighted the following points regarding *J. curcas* yield:

- The earlier reported figures of *Jatropha curcas* seed yield exhibited a very wide range (0.4–12 t ha^{-1} yr^{-1}) and are not coherent mainly because of incorrect extrapolation of annual yields of individual trees to ha^{-1} yr^{-1} yields;
- The effect of spacing, canopy management and crown form on the yield is not known;

- There are positive trends in the influence of both average annual rainfall and age on seed yield. Mainly the upper boundary of the yield as a function of the rainfall is interesting and shows a clear difference between low rainfall and high rainfall regimes;

- *Jatropha curcas* has not yet undergone a careful breeding programme with systematic selection and improvement of suitable germplasm. This is why it can still be considered a wild plant that exhibits great variability in productivity between individuals;

- Where good sites (good soil and average annual rainfall of 900–1200 mm) and optimal management practices are used, 5 t dry seed ha^{-1} yr^{-1} can be achieved;

- *Jatropha curcas* is a hardy and highly adaptable plant that can grow in marginal soils from an average annual rainfall of 250 mm. As such *J. curcas* is acceptable to reclaim wasteland. However, there is uncertainty about its ability to produce ecologically and socio-economically viable amounts of energy in these harsh situations; and

- Average shell:kernel ratio on mass basis of *J. curcas* seeds is approximately 37:63. The kernel mainly contains crude fat and protein and has an average calorific value of 30.4 MJ kg^{-1}. The shell is mainly composed of fibre and has a calorific value of 19.4 MJ kg^{-1}. Based on these figures the average oil content of dry seed on mass basis is 34.4%.

Table 2.1 represents published data on *J. curcas* yield. Variations in yield and plantation condtitions are recorded.

Table 2.1 Data yield of *Jatropha curcas* seeds (t/ha) from different countries, patterns, age and growing conditions.

Yield (t/ ha)	Country	Pattern	Age	Condition	Reference:
0.8 – 1.0	Mali	Hedge	–	–	HENNING, 1998
> 1		Crop	1 – 2 year	500 – 600 mm y^{-1}	EULER and GORRIZ, 2004
3.2 – 4.1	India	Crop	1st year	Rain fed marginal lands	LAL *et al.,* 2004
0.335	Brazil	Crop	12 months	Drip irrigation, spacing 4x3 m, density 833 plant ha^{-1}	MATTANA SATURNINO *et al.,* 2005
0.19	Brazil	Crop	9 months	Drip irrigation, spacing 8x2 m, density 625 plant ha^{-1}	MATTANA SATURNINO *et al.,* 2005
0.056	Brazil	Crop	7 months	Drip irrigation, spacing 8x2 m, density 625 plant ha^{-1}	MATTANA SATURNINO *et al.,* 2005
0.6	India	Crop	2.5 year	Marginal solis, density 833 plant ha^{-1}	GOSH *et al.,* 2007
1.45	India	Crop	2.5 year	Marginal solis, density 1677 plant ha^{-1}	GOSH *et al.,* 2007
3.0	Indonesia	Plantation	1st year	–	MANURUNG, 2007
1.25	Guatemala	Plantation	1st year	Spacing 2.5 x 2.5 m, Fertilizer and 800 mm irrigation during the 6 dary months and 6 months of rain fall 4000 mm. in^{-1}	OUWENS *et al.,* 2007
4.5	Nicaragua	Plantation	4 years	Best field condtion	OUWENS *et al.,* 2007

1.1.8 Research and development programmes for *Jatropha curcas* yield improvement

The productivity of *Jatropha curcas* has been reported to be less economically beneficial to farmers due to lower productivity and non-availability of protocols. Therefore, efforts have been made to enhance the productivity and development of location specific protocols. In India a network on *Jatropha* has been initiated with major objectives of: selection of superior planting material; standardization of propagation techniques (micro- and macro-propagation); standardizing agricultural techniques; establishment of model plantations; tree improvement; detoxification of seed meal; development of pre-processing; and processing equipment (KUREEL, 2006). Advance lines of *Jatropha curcas* with high oil content; high yield; a more drought tolerante character; resistance to insect-pests; and diseases have been identified. These traits were transferred by crossing with specific characters in order to develop high yielding varieties through hybridization. To develop quality planting material, techniques for mass multiplication of superior quality planting material were developed (KUREEL, 2006).

1.2 BIOFUELS AND BIODIESEL

1.2.1 Introduction

Petroleum-based fuel reserves are limited and on the verge of reaching their peak production (DEMIRBAS, 2009). The amount of greenhouse gases in the atmosphere is rising as a consequence of human activity. These anthropogenic emissions are resulting in increased global atmospheric temperatures, so by the end of the Century the planet's average temperature could increase by 6.4 degrees Celsius. Bio-fuels are generally considered as offering many advantages, including sustainability, reduction of greenhouse gas emissions, regional development, social structure and agriculture, security of supply (REIJNDERS, 2006). These factors make renewable energy resources very attractive (OZCIMEN, 2004; FERNANDO *et al.,* 2006; JEFFERSON, 2006; DEMIRBAS, 2007, 2009). Therefore there is a growing trend towards employing

modern technologies and efficient bio-energy conversion using a range of biofuels, which are becoming, cost-wise, competitive with fossil fuels (PUHAN *et al.,* 2005).

Biodiesel (Greek, bio, life + diesel from Rudolf Diesel) refers to a diesel equivalent, processed fuel derived from biological sources (DEMIRBAS, 2009). Biodiesel is an alternative fuel for diesel engines. It is produced by chemically reacting a triglyceride (vegetable oil or animal fat) with a short-chain alcohol such as methanol or ethanol. The transesterification reaction requires a catalyst, usually a strong base, such as sodium or potassium hydroxide (SARIN *et al*., 2007, HANHA *et al.,* 2009).

1.2.2 Biodiesel sources and main biodiesel crops

Biodiesel can be produced commercially from a variety of oils and fats:

- Animal fats: tallow, lard, grease, poultry fats and fish oils; and
- Vegetable oils.

There are more than 350 oil-bearing crops that have been identified (GOERING *et al*., 1982). Biodiesel is made from a variety of natural oils such as rapeseed oil and soybean oil. Rapeseed oil dominates the growing biodiesel industry in Europe. In the United States, biodiesel is made from soybean. There are many other feedstock candidates, including other oilseed crops (SHEEHAN *et al*., 1998a). DEMIRBAS (2009) categorized oil species for biodiesel production as follows:

(a) *Major oils,* Coconut (copra), corn (maize), cottonseed, canola (a variety of rapeseed), olive, peanut (groundnut), safflower, sesame, soybean, and sunflower;

(b) *Nut oils,* Almond, cashew, hazelnut, macadamia, pecan, pistachio and walnut;

(c) *Edible oils,* Amaranth, apricot, argan, artichoke, avocado, bay laurel, beech nut, ben, Borneo tallow nut, carob pod (algaroba), cohune, coriander seed, false flax,

grape seed, hemp, kapok seed, lallemantia, lemon seed, macauba fruit (*Acrocomia sclerocarpa*), meadowfoam seed, mustard, okra seed (hibiscus seed), perilla seed, pequi, (*Caryocar brasiliensis* seed), pine nut, poppy seed, prune kernel, quinoa, ramtil (*Guizotia abyssinica* seed or Niger pea), rice bran, tallow, tea (camellia), thistle (*Silybum marianum* seed), wheat germ; and

(d) *Inedible oils,* Algae, babassu tree, copaiba, honge, jatropha, jojoba, karanja or honge, mahua, milk bush, nagchampa, neem, petroleum nut, rubber seedtree, silk cotton tree, tall, castor, radish, and tung.

From the list above, algae emerged as a promising candidate for biodiesel production. SHEEHAN *et al*. (1998a) and GRESSEL (2008) stated that algae can grow practically in every place where there is enough sunshine. Some algae can grow in saline water. The most significant difference of algal oil is in the yield and hence its biodiesel yield. According to some estimates, the yield (per ha) of oil from algae is over 200 times the yield from the best-performing plant/vegetable (**Table 2.2**).

Table 2.2 Biodiesel sources and main biodiesel crops (adapted from GRESSEL, 2008).

Crop	Oil yield (l/ha)	Land area needed (million ha)
Maize	172	462
Soybean	446	178
Oilseed rape	1190	67
Jatropha curcas	1892	42
Oil palm	5950	13
Algae Cyanobacteria[a]	59000	1.3
Algae Cyanobacteria[b]	137000	0.6

[a]Containing 30% oil; [b]Containing 70% oil.

1.2.3 Advantages and disadvantages of biodiesel

1.2.3.1 Advantages of biodiesel

Biodiesel is environmentally friendly compared with gasoline and petroleum diesel. The advantages of biodiesel as a diesel fuel are its portability, ready availability, renewability, higher combustion efficiency, lower sulfur and aromatic content, higher cetane number, and higher biodegradability (DEMIRBAS, 2009).

The following advantages are summarized from DEMIRBAS (2009):

(1) Availability and renewability of biodiesel:

- Biodiesel can be made from domestically produced, renewable oilseed crops such as soybean, rapeseed, and sunflower;
- The risks of handling, transporting, and storing biodiesel are much lower than those associated with petrodiesel;
- Biodiesel is the only alternative fuel in which low-concentration biodiesel–diesel blends run on conventional unmodified engines. It can be stored anywhere that petroleum diesel fuel is stored; and
- Biodiesel is safe to handle and transport because it is as biodegradable as sugar and has a high flash point compared to petroleum diesel fuel. Biodiesel can be used alone or mixed in any ratio with petroleum diesel fuel.

(2) Lower emissions from biodiesel

In cities across the globe, the personal automobile is the single greatest polluter, as emissions from millions of vehicles on the road contribute to a worldwide problem. The biodiesel impacts on exhaust emissions vary depending on the type of biodiesel and on the type of conventional diesel. The commercial biodiesel fuel significantly reduced

particulate matter (PM) exhaust emissions (75–83%) compared to the petrodiesel base fuel. However, nitrogen oxide (NOx) exhaust emissions increased slightly with commercial biodiesel compared with the base fuel. The chain length of the compounds had little effect on NOx and PM exhaust emissions, while the influence was greater on hydrocarbon and CO, the latter being reduced with decreasing chain length. Non-saturation in the fatty compounds causes an increase in NOx exhaust emissions (DEMIRBAS, 2009).

(3) Biodegradability of biodiesel

Biodegradability of biodiesel has been proposed as a solution for the waste problem. Biodegradable fuels such as biodiesels have an expanding range of potential applications and they are environmentally friendly. Therefore there is growing interest in degradable diesel fuels that degrade more rapidly than conventional disposable fuels. Biodiesel is non-toxic and degrades about four times faster than petrodiesel. Its oxygen content improves the biodegradation process, leading to a decreased level of quick biodegradation (DEMIRBAS, 2009).

(4) Higher lubricity

Biodiesel methyl esters improve the lubrication properties of the diesel fuel blend. Fuel injectors and some types of fuel pumps rely on fuel for lubrication. Biodiesel reduced long term engine wear in test diesel engines to less than half of what was observed in engines running on current low sulfur diesel fuel. Lubricity properties of fuel are important for reducing friction wear in engine components normally lubricated by the fuel rather than crankcase oil (DEMIRBAS, 2009).

(5) Engine performance evaluation using biodiesel

Biodiesels are mono-alkyl esters containing approximately 10% oxygen by weight. The oxygen improves the efficiency of combustion, but it takes up space in the blend and therefore slightly increases the apparent fuel consumption rate observed while operating an engine with biodiesel. The high combustion temperature at high engine speed becomes the dominant factor, making both heated and unheated fuel to acquire the same temperature before fuel injection. Various methods of using vegetable oil (Jatropha oil) and methanol such as blending, transesterification and dual fuel operation were studied experimentally. Brake thermal efficiency was better in the dual fuel operation and with the methyl ester of Jatropha oil as compared with the blend. It increased from 27.4% with neat Jatropha oil to a maximum of 29% with the methyl ester and 28.7% in the dual fuel operation (DEMIRBAS, 2009).

1.2.3.2 Disadvantages of biodiesel as diesel fuel

According to DEMIRBAS (2009) the disadvantages of biodiesel are:

- The major disadvantages of biodiesel are its higher viscosity, lower energy content, higher cloud point and pour point, higher nitrogen oxide (NOx) emission, lower engine speed and power, injector coking, engine compatibility, and high price;

- The biodiesels on the average decrease power by 5% compared to that of diesel at rated load. The maximum torque values are about 21.0 Nm at 1500 RPM for diesel fuel, and 19.7 Nm at 1500 RPM for biodiesel. The torque values of commercial diesel fuel are greater than those of biodiesel. Peak torque applies less to biodiesel fuels than it does to No. 2 diesel fuel but occurs at lower engine speed and generally its torque curves are flatter;

- The specific fuel consumption values of biodiesel are greater than those of commercial diesel fuel;

- The effective efficiency and effective pressure values of commercial diesel fuel are greater than those of biodiesel;

- Important operating disadvantages of biodiesel in comparison with petrodiesel are cold start problems, the lower energy content, higher copper strip corrosion and fuel pumping difficulty from higher viscosity; and

- Fuel consumption at full load condition and low speeds generally is high. Fuel consumption first decreases and then increases with increasing speed. The reason is that, the produced power at low speeds is low and the main part of fuel is consumed to overcome engine friction.

1.2.4 Biodiesel vs. petroleum diesel

Biodiesel is technically competitive with, or offers technical advantages compared with conventional petroleum diesel fuel. The vegetable oils can be converted to their methyl esters via a transesterification process in the presence of a catalyst. The bio-diesel esters are characterized for their physical and fuel properties including density, viscosity, iodine value, acid value, cloud point, pour point, gross heat of combustion and volatility. The biodiesel fuels produced slightly lower power and torque, and higher fuel consumption than diesel fuel. Biodiesel is better than diesel fuel in terms of sulphur content, flash point, aromatic content and biodegradability (MA and HANNA, 1999). The cost of biodiesels varies depending on the base stock, geographic area, variability in crop production from season to season, the price of crude petroleum. Biodiesel has over double the price of petroleum diesel. The high price of biodiesel is in a large part due to the high price of the feedstock (ACHTEN, 2008).

1.2.5 Oil properties of *Jatropha curcas*

The composition and characteristics of crude *J. curcas* oil are given in **Tables 2.3 and 2.4**. The oil quality is dependent on the interaction of environment and genetics. As for seed size, seed weight and oil content also for the oil quality, it is beleaved that the environmental conditions have a larger impact than the genetics (ACHTEN, 2008). *Jatropha curcas* oil (**Figure 2.1**) contains about 14% free fatty acids (FFA), which is

beyond the limit of 1% FFA level that can be converted into biodiesel by trans-esterification using an alkaline catalyst (TIWARI *et al*., 2007). The fatty acid profile of *J. curcas* is given in (**Table 2.5**). *Jatropha curcas* oil contains more than 75% unsaturated fatty acid, which is reflected in the pour and cloud point of the oil. The fatty acid composition of *J. curcas* oil is dominated by oleic acid and linoleic acid. The maturity stage of the fruits at the moment of collection is reported to influence the fatty acid composition of the oil (ACHTEN, 2008).

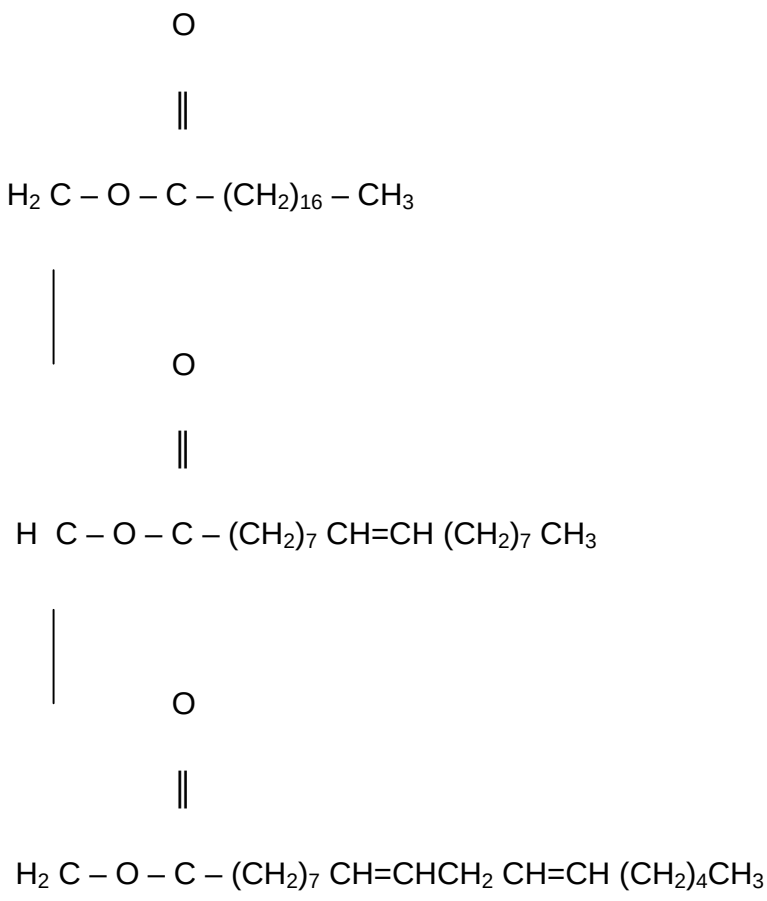

Figure 2.1 Organic structure of *Jatropha curcas* oil (JONGSCHAAP *et al.,* 2007).

Table 2.3 Proximate composition (%) of *Jatropha curcas* seed flour (AKINTAYO, 2004).

Assay	Value (%)
Crude fat	47.3
Crude fibre	12.0
Moisture	5.5
Ash	27.2
Carbohydrate (by difference)	8.0

Table 2.4 Physico-chemical characteristics of *Jatropha curcas* seed oil (adapted from AKINTAYO, 2004 and DEMIRBAS, 2009).

Parameter	Value
Colour	Light yellow
Free fatty acid (mg/g)	1.76
Acid value (mg KOH/g)	5
Saponification value (mg KOH/g)	198.8
Iodine value (mg iodine/g Wijs)	105
Mean molecular mass	281.6
Unsaponifiable matter (%)	10.8
Refractive index (25 ℃)	1.5
Specific gravity (25 ℃)	0.9
Hydroxyl value	2.15
Acetyl value	16
Viscosity (30 ℃) cSt	17.1

Table 2.5 Fatty acid composition of *Jatropha curcas* oil. (ADEBOWALE and ADEDIRE, 2006).

Fatty acid	Systemic name	Formula	Structure [a]	Weight (%)
Palmitic	Hexadecanoic	$C_{16}H_{32}O_2$	16:0	11.3
Stearic	Octadecanoic	$C_{18}H_{36}O_2$	18:0	17.0
Arachidic	Ecosanoic	$C_{20}H_{40}O_2$	20:0	4.7
Oleic	*cis*-9-Octadecenoic	$C_{18}H_{34}O_2$	18:1	12.8
Linoleic	*cis*-9,*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	18:2	47.3

[a]Carbons in the chain: number of double bonds

1.2.6 Oil extraction

The choice of extraction method is dependent on the intended scale of the activity. The two extraction procedures, mechanical and chemical, are quite well established, although there is still scope for further research. Both of them have their advantages and disadvantages with respect to scale suitability, centralization, extraction efficiency and environmental and health risks. Further research should investigate efficiency, improvement of mechanical oil extraction, the applicability of alternative solvents such as supercritical CO_2, bio-ethanol and isopropyl alcohol and their economic viability (ACHTEN, 2008).

1.2.7 Biodiesel production process from *Jatropha curcas*

Biodiesel is manufactured by the transeterification of oils with methanol in the presence of a catalyst, such as alkalis (KOH, NaOH) or their corresponding alkoxides (MA and HANNA, 1999). However, *Jatropha curcas* oil has an high content of FFA (**Table 2.5)** therefore cannot be directly used with an alkali catalyzed by homogeneous acids, such as sulphuric acid, phosphoric acid, sulfonic acid (LU *et al.,* 2008). Glycerol is an important by-product. It can be burned for heat or be used as feedstock in the cosmetic industry (ACHTEN, 2008). Although the transesterification process is quite straight forward, the genetic and environmental background of the produced oil might require

modification of the input ratios of the alcohol reagent and reaction catalyst as well as alterations to reaction temperature and time, in order to reach optimal bio-diesel production (ACHTEN, 2008). The fuel properties of *J. curcas*-oil, -biodiesel and conventional diesel are given in (**Table 2.6**).

Table 2.6 Fuel properties of *Jatropha curcas*-oil, -biodiesel and conventional diesel. (TIWARI *et al.* 2007; VYAS *et al.* 2008).

Property	Unit	*J. curcas* oil	*J. curcas* biodiesel	Diesel	Biodiesel standards	
					ASTM D 6751-02	DIN EN 14214
Density at 15 ℃	Kg m^{-3}	940	880	850	-	860 – 900
Viscosity at 15 ℃	Mm2 s^{-1}	24.5	4.80	2.60	1.9 – 0.6	3.5 – 5.0
Flash point	℃	225	135	68	> 130	> 120
Specific gravity at 15℃	Kg/l	0.912	0.862	0.846		
Cloud point	℃	20	8	-8	-3 - 12	-
Fire point	℃	136	123	63	-	-
Pour point	℃	4	2	-20	-	-
Aniline point	℃	135	135	68	-	-
Water content	℃	1.4	0.025	0.02	< 0.03	< 0.05
Ash content	%	0.8	0.012	0.01	< 0.02	<0.02
Carbon residue	%	1.0	0.20	0.17	-	<0.30
Acid value	Mg KOH g^{-1}	28.0	0.40	—	< 0.80	< 0.50
Cetane number		57	57	50	48-65	
Calorific value	MJ kg^{-1}	38.65	39.23	42	-	—

1.3 THE STUDY SITE

1.3.1 Location, planting dates, and plant density

The present experiments were conducted in a monoculture plantation at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga) Pietermaritzburg, South Africa, (30° 41' E, 29° 67' S and 781 m asl). This plantation was established with the overall aim of building capacity for small-scale farmers and poor communities to implement agroforestry systems that enable them to increase production while simultaneously rehabilitating and improving the land resources (EVERSON and EVERSON, 2006). The plantation was established from seeds. The seeds were obtained from collections made by the Department of Agriculture at the Owen Sitole College of Agriculture. The original seeds came from Malawi. Four thousand five hundred seeds were planted directly into small plastics bags in a nursery with an automatic watering system. Three seeds were planted in bags in order to obtain the required 2500 *J. curcas* seedlings. By the end of January 2005 the seedlings had developed to a stage where they could be moved out of the greenhouse to allow them to harden before planting. The plants were planted in two different dates Feburuary 2005 and May 2007. The total area of the trail is 2.9 ha (265 m long x 110 m wide); the spacing between plants is 3 x 3 m giving a plant population of 1111 trees ha^{-1} (EVERSON and EVERSON, 2006).

1.3.2 Meteorological data during the study period

Figure 2.2 represents meteorological data recorded from the Weather Station at the University of Kwazulu-Natal Research Station, Pietermaritzburg, South Africa. The data were recorded during 2007 when the experiments for this thesis were undertaken. The data are monthly means of solar radiation, total rain, average air temperature, relative humidity and wind speed.

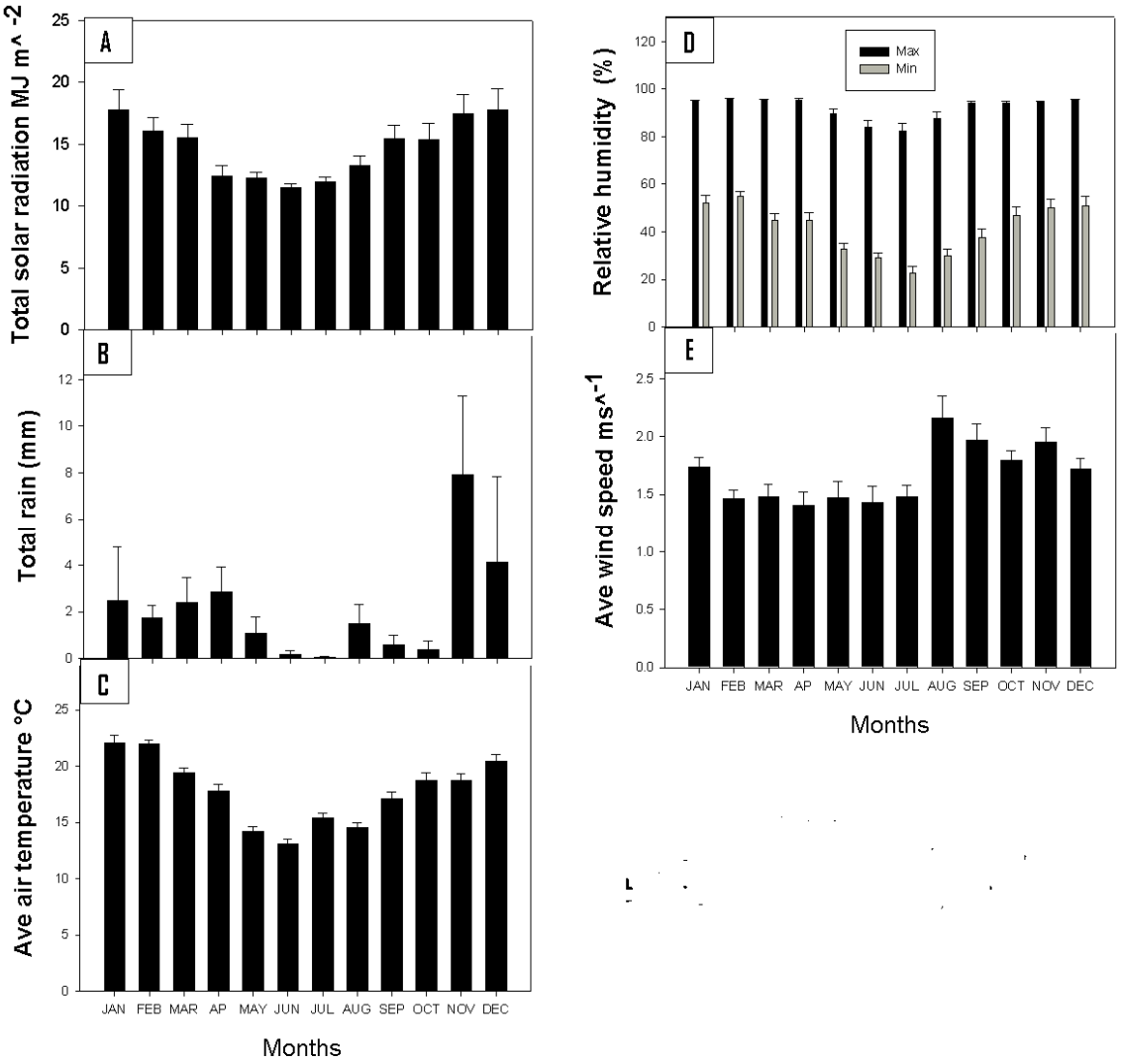

Figure 2.2 Meteorological data during the study period at Ukulinga Agricultural Research Station, University of Kwazulu-Natal, Pietermaritzburg, South Africa, (30° 41' E, 29° 67' S and 781 m asl). (A) Solar radiation; (B) Total rain; (C) Average air temperature; (D) Relative humidity; and (E) Average wind speed. Source (Weather Station of the Agricultural Research Station Ukulinga, University of Kwazulu-Natal, Pietermaritzburg, South Africa.

1.3.3 Irrigation, weeding and mulching

The trial had an irrigation system installed to ensure survival of the trees during the early establishment period. Shortly after planting, the trial was invaded by weeds which were controlled by a broad-spectrum herbicide (Round Up) to clear all the unwanted vegetation. In these plots care was taken to first cover the trees with buckets before spraying, to avoid drift of the herbicide onto the trees. Mulches helped to keep the soil well aerated by reducing soil compaction when raindrops hit the soil. They help maintain a more uniform soil temperature and promote the growth of soil micro-organisms and earth worms. Mulches eliminate mowing around trees and provide a physical barrier that prevents damage from mowers. Following periods of mowing in order to control weeds at the trial site, it was found that there was an abundance of mulch (grass and weed cuttings). The mulch was applied to the base of the trees to reduce weed regrowth and conserve moisture (EVERSON and EVERSON, 2006).

2.3.4 Pests and diseases

In the early stage, ten months from planting, insect damage was detected on the trees. The initial indications of the problem were loss of condition in the trees through wilting and defoliation. Holes in the leaves were detected, and upon closer inspection it was found that the defoliation was being caused by insect damage. Samples of the insects were collected and sent for analysis, and were found to be a species of flea beetle. The specimen was identified as a member of the Chrysomelidae, Alticinae family, and was recognized as an *Aphtona* species. These beetles are known to preferentially feed on Euphorbiaceae. Specialist advice on controlling the insect was sought and the solution was found to be a combination of PREV-AM and CYPERFOS 500 EC insecticides (EVERSON AND EVERSON, 2006). A further symptom of insect damage was blackened tips to the branches, appearing as if the trees had been scorched by fire. At this stage the trees had suffered complete defoliation with not a single leaf evident. Even the emerging buds were eaten off by the insects before being able to emerge. However, after spraying with the above insecticides, there was a very rapid positive response. Within 48 hours of spraying the insects had disappeared and the emergence of buds and new leaves was noted.

Weekly follow-up spraying is recommended until there is no sign of the insects (EVERSON AND EVERSON, 2006).

1.4 PROBLEM STATEMENT

Declining availability of fossil fuels is driving a search for alternative sources of energy. Biofuels offer promise, but are controversial because of the large land area required for production, potential for competition with food production, and their marginal economic viability in the absence of subsidies (PARFIT, 2004; GRESSEL, 2008). These potential negative impacts could be reduced and profitability increased if production could be made more efficient. *Jatropha curcas* L. is a crop with a high potential for biodiesel production in semi-arid and arid conditions. It is a drought-resistant perennial plant that can grow on marginal lands, thus it does not compete with food crops (HELLER, 1996; AUGUSTUS *et al*., 2002; AZAM *et al*., 2005; SHARMA, 2006). In recent years, the production of *J. curcas* has been widely promoted by private enterprises as one of the most viable candidates for biodiesel production in Africa. However, farmers will not invest if they do not have a minimum guaranteed market (TOMOMATSU and SWALLOW, 2007). On the other hand, Industry will not invest if there is no reliable and secure feedstock supply. The challenge, however, is whether sufficient yield can be produced to make the enterprise commercially viable. In South Africa the government has put a moratorium on all *J. curcas* cultivation outside research areas (GUSH, 2006). This is largely due to insufficient knowledge relating to this species, such as its invasive potential, water use, production potential and maintenance requirements (OPENSHAW, 2000; AGUSTUS *et al*., 2002; GUSH 2006).

In late 2006, Verus Farming and Investments (Pty) Ltd., which, is an agribusiness company planning to grow *J. curcas* on a large scale in plantations in the southern African region, approached the Research Centre for Plant Growth and Development, University of KwaZulu-Natal, Pietermaritzburg, South Africa. This was to initiate research and development to improve *J. curcas* seed production. There were several

potential avenues for the research to be directed towards increased seed production. However, the general areas of the research were outlined as:

- Hormonal regulation of vegetative and reproductive growth;
- Pollination improvement;
- Agronomic improvement; and
- An intensive breeding programme.

This PhD study reports on hormonal regulation of growth and pollination.

1.5 AIMS AND OBJECTIVES

The overall aim of this study was to develop approaches to improve seed production of *J. curcas* L. to become commercially viable. Thus, the specific objectives of this study were:

- To investigate the effects of plant growth regulators, smoke applications and nitrogen salts on germination and seedling growth;
- To report in detail on the pollination system, test for inbreeding depression, observe the flower visitors and investigate their contribution to fruit set and yield;
- To investigate pollen viability, *in vitro* pollen germination and pollen tube growth, the influence of type of pollination on the success of fertilization through *in vivo* studies;
- To investigate the response of *J. curcas* to winter and summer manual pruning;
- To investigate the influence of plant growth regulators in promotion of lateral branching; and
- To investigate the influence of plant growth regulators on flowering, fruit set, seed oil content and oil quality.

1.6 GENERAL OVERVIEW

Chapter 2

- Provides a background for the strategies used in the study to assist in understanding and interpreting the results;
- Provides a general background on plant pollination and its potential role in crop improvement. In this regards it covers the role of insects and specifically bees;
- Covers the aspects of pollen biology and pollen biotechnology and its potential role in crop development;
- Reviews some of the work done on different crops as result of pollination management and pollen biotechnology application;
- Since this study includes the usage of a range of PGRs for different purposes (germination, seedling vigour, pollen germination, promotion of branching, flowering, fruit set, seed oil content and oil quality), this Chapter was intended to provide a full background about PGRs, their nature and biosynthesis, with emphasis on their agricultural use. Some of the PGRs in this review were not used in the experiments but, as the PGRs interact together, they were included to give a complete picture; and
- Reviews on smoke application (aerosol smoke and smoke water) in germination, seedling vigour and their potential role in agricultural and horticultural crop improvement.

Chapter 3 reports on the response of *J. curcas* to summer and winter manual pruning. The results showed that manual pruning under both conditions increased significantly the number of branches per plant. However, there were no significant differences in number of branches between winter and summer pruning. Winter pruning, however, produced significantly wider crown diameters compared with the

control and summer pruning. Both treatments produced significantly less fruits/per plant in the subsequent season.

Chapter 4 investigated the effect of foliar application of PGRs on branching of *J. curcas* as possible substitution to manual pruning. The results indicated that a single foliar application of PGRs under field conditions can be an alternative method to MP for increasing the number of lateral branches.

Chapter 5 details the pollination system in *J. curcas* by addressing four basic questions: (1) What is the relative importance of cross- and self-pollination for fruit set?, (2) Do honeybees contribute effectively to fruit set?, (3) Is *J. curcas* pollen-limited by pollen availability?, and (4) Do self-fertilized progeny of *J. curcas* experience inbreeding depression? The results provided empirical evidence that honeybees are effective pollinators of *J. curcas*. There are strong suggestions that promotion of cross-pollination does not have to be a top priority in orchard management for fruit yield.

Chapter 6 compares different stains to test for pollen viability in *J. curcas*. Tests for *in vitro* pollen germination and pollen tube growth in *J. curcas*. The comparison is for pollen grains from the different types of flowers in this plant, in response to different germination media. Tests for pollen-pistil interaction by comparing *in vivo* pollen germination and pollen tube growth following self- and cross-pollination. The results showed that hermaphrodite flower pollen was less viable compared to that of male flowers. There was strong evidence to suggest that type of pollination has no influence on the success of fertilization in *J. curcas.*

Chapter 7 is an evaluation of the subsequent influences of foliar application of different PGRs used as chemical pruning agents on flowering, fruit set, seed oil content and oil quality. The results indicated that foliar application of PGRs can be used in *J. curcas* to increase seed production and seed oil content.

Chapter 8 is an investigation on germination, seedling growth and vigour in *J. curcas* in response to aerosol smoke, smoke water, potassium nitrate, naphthalene acetic acid and indole 3-butyric acid. The findings from this investigation suggested the possibility of applying these treatments to produce quality seedlings and a good crop.

Chapter 9 provides a general conclusion and recommendations.

Chapter 10 lists the cited references.

2 Literature review

2.1 POLLINATION

2.1.1 Introduction

Pollination is the transfer of pollen grains from the anther to the stigma. In seed plants pollination is a prerequisite for fruit and seed development and is the basis of genetic exchange between plants and recombination within plants. Pollination is a critical factor for sustainable agriculture and for commercial production of hybrid seeds. The various aspects of pollination biology have been reviewed by FAEGRI and VAN DER PIJL (1979), REAL (1983), RICHARDS (1986), BAWA *et al.* (1993), FREE, (1993) and ROUBIK (1995). The techniques of pollination biology have been covered comprehensively by JONES and LITTLE (1983), DAFNI (1992), KEARNS and INOUYE (1993), DAFNI and FIRMAGE (2000).

2.1.2 Types of pollen transfer

- Autogamy: Is the transfer of pollen grains from the anther to the stigma of the same flower.
- Geitonogamy: Is the transfer of pollen grains from the anther to the stigma of another flower of the same plant or another plant of the same clone.
- Xenogamy: Is the transfer of pollen grains from the anther to the stigma of a different plant.
- Allogamy: Is the transfer of pollen grains from the anther to the stigma of another flower of the same or another plant (include both geitonogamy and xenogamy).

The major advantage of self pollination is reproductive assurance. However, continued self pollination over many generations results in inbreeding depression. For effective self pollination (autogamy), the flowers have to be bisexual, anther

dehiscence and stigma receptivity have to be synchronous, and the position of the stigma and anthers should be close to one another so that pollen grains readily come into contact with the stigma soon after anther dehiscence (SHIVANNA, 2003). In most crop species the flowers open and sex organs in the flower become visible; such flowers are called chasmogamous. In some plants, a proportion of flowers remains closed, such flowers are termed cleistogamous (LORD, 1981). Many species produce both chasmogamous and cleistogamous flowers depending on the environmental conditions, especially temperature and light duration. In cleistogamous flowers, self-pollination is the norm, whereas chasmogamous flowers may be predominantly self-pollinated or cross-pollinated. Some species bearing chasmogamous flowers, such as *Utriculata* (KHOSLA *et al*., 2000), also show obligate self-pollination. Pollen grains germinate *in situ* inside the anthers before dehiscence. Because of the physical contact between the stigma and the anthers, dehiscence of anthers results in deposition of *in situ* germinated pollen mass of the stigma before opening of the flower. This is often referred to as pre-anthesis cleistogamy (LORD, 1981). A unique method of self-fertilization, termed internal geitonogamy has been reported in some members of Malpighiaceae (ANDERSON, 1980) and Callitrichaceae (PHILBRICK and ANDERSON, 1992). Cross pollination results in genetic heterogeneity and thus cross-pollinated species show wider adaptations. Cross pollination involves considerable wastage of resources because of its uncertainty and thus cross pollinated species have to produce much more pollen to compensate for the wastage (SHIVANNA, 2003).

2.1.3 Breeding system

A breeding system is defined as all aspects of sex expression that affect the relative genetic contributions to the next generation of individuals within a species (WYATT, 1983). The system includes pollination mechanism and pollen movements. Traditionally, breeding systems have been treated in relation to the mechanism(s) which promote(s) or reduce(s) outcrossing (DAFNI, 1992). Fruit- and seed-set are especially dependant on successful pollination when the species under consideration cannot or must not be automatically selfed (WESTERKAMP and GOTTSBERGER, 2000). The proportion of outcrossing and selfing in a population depends, in part, on self-incompatibility mechanisms, floral development, and pollinator behaviour

(WYATT, 1983). However, if pollen is proximal to the stigma and the stigma is receptive when the pollen is viable, then autonomous selfing may occur frequently (LLOYD and SCHOEN, 1992). Additionally, facilitated selfing may be high in plants with many inflorescences and where both female and male phases are mature because pollinators may forage longer among flowers of the same plant (HARDER and BARRETT, 1995). Because high levels of selfing in a xenogamous species could lead to inbreeding depression, any floral trait that maintains outcrossing is likely to be advantageous (LANDE and SCHEMSKE, 1985).

2.1.3 1 Outbreeding sytems

- Dichogamy: In dichogamous species, anther dehiscence and stigma receptivity are temporarily separated. The anther and the stigma mature at different periods; the time gap between the two may vary from one day to many days. In some species, the anthers dehisce before the stigma becomes receptive (protandry) and thus autogamy is prevented. In certain other species, the stigma becomes receptive before the anthers dehisce (protogyny) (LLOYD and WEBB, 1986; BERTIN and NEWMAN, 1993).

- Herkogamy: herkogamous species show spatial separation of the anthers and the stigma. Their relative position is such that autogamy cannot occur (WEBB and LLOYD, 1988). The stigma often projects beyond the level of the anthers, and therefore the pollen level of the anthers, and therefore the pollen of the same flower cannot land on the stigma.

- Self-incompatibility: In many species, self pollination does not result in fertilization. This is because pollen germination on the stigma or the growth of pollen tubes in the stigma or style is inhibited. For effective fertilization, pollen has to come from another plant. Self incompatibility (SI) is genetically controlled and widespread in flowering plants (DE NETTANCOURT, 1977).

- Dicliny: In diclinous species, the flowers are unisexual. In some species such as *Cucurbita*, male and female flowers are borne on the same plant (monoecious), in others, such as papaya and cannabis, male and female flowers are borne on different plants (dioecious).

Only the dioecious form of dicliny prevents both autogamy and geitonogamy. In most of the species which bear bisexual flowers, generally a combination of self- and cross-pollinations occurs to different degrees; often species can be predominantly an inbreeder or outbreeder (RICHARDS, 1986; KOUL *et al*., 1993). Some species exhibit more than one outbreeding device, such as *Anthocercis gracilis* which shows protogyny as well as self-incompatibility (STACE, 1995).

2.1.3.2 Pollen:ovule ratio

The number of pollen grains produced for each ovule of a flower has been reported to reflect the breeding system of the species (CRUDEN, 1977). The pollen:ovule ratio gives an estimate of the outcrossing level.

2.1.4 Mode of pollination

Pollination agents are: biotic (many species of animals) and abiotic (wind and water) (REAL, 1983; DAFNI, 1992). Insects are considered the original pollinators of early flowering plants. Those species which use non-insect pollinators, are believed to be derived (ENDRESS, 1994).

2.1.4.1 Anemophily

Wind is the major of the two abiotic pollinating agents (WHITEHEAD, 1983). Wind pollination, termed anemophily, is prevalent in dry environment open grasslands, savannahs and semiarid areas. According to KEVAN, 1997; SHIVANNA, 2003 anemophilous flowers and plants have the following characteristics:

- Flowers are generally small, inconspicuous, emit no scent and do not produce nectar;
- Pollen producing flowers are often clustered as catkins in anemophilous species;

- Generally have well-exposed stamens; the anthers are generally suspended from long filaments and hang freely from the flowers;

- The stigmas are generally dry, have a large surface, and are often feathery; and

- The plants produce an enormous amount of pollen; this is achieved by an increase in the size of anthers or in number of stamens per flower. The distance which the pollen has to travel to reach a stigma depends on pollen longevity and prevailing wind currents.

2.1.4.2 Zoophily

The number of species pollinated by animals far exceeds that pollinated by wind and water and the adaptations that zoophilous plants show are also very diverse (ROUBIK, 1995). Zoophilous species develop effective floral devices to attract pollinating agents. Floral attractants, rewards and flower modification largely determine the class of animal species able to visit the flowers, and the degree of species specificity (FAEGRI and VAN DER PIJL, 1979). Among the animals, insects are the most important pollinators (entomophily); of these the most common insects are bees (melittophily), beetles (cantharophily), moths (phalaenophily) and butterflies (psychophily). In many instances pollinators transport pollen on particular parts of their body; they are basically of three types: nototribic (on their back), sternotribic (on the underside), and pleurotribic (on the flanks) (SHIVANNA, 2003). Bees are the prime pollinators; they are involved in pollination of most field and orchard crops. Social bees are especially versatile as they are able to exploit a broad range of different flower forms. Bees live on nectar; they collect pollen also to feed their larvae. Bees are good at recognizing colours, scents and contours. Bees can perceive ultraviolet light, but they cannot visualize shades of red, which appear black to them. Bee-pollinated flowers are showy, brightly coloured (mostly yellow) and often exhibit distinctive markings on the petals (honey guides); honey guides help the bees reach the source of nectar easily. Both nectar and pollen serve as rewards for the bees. The nectary in bee-pollinated flowers is usually situated at the base of the corolla tube and is therefore accessible only to those bee species with a tongue of

the right length. Pollen adheres to the bristles of the bees or the bees pack it in special baskets on their legs (SHIVANNA, 2003).

2.1.5 Floral attractants and rewards

Zoophilous plants have to fulfill the following requirements to attract pollinators:

- Advertise the presence of a reward;
- Supply some reward; and
- Position the anthers and stigmas so that they come into contact with the body of the pollinator to facilitate transfer of pollen.

The attractants/advertisements are largely visual and olfactory. Visual advertisements include flower colour, size and shape. Colour differences between flowers allow pollinators to discriminate between species and varieties. Flowers of many species show a colour pattern (nectar guides) in the UV range which directs the pollinators to the floral reward in such a way that pollination is assured (SHIVANNA, 2003). The colour of many flowers in many species changes in response to pollination (MATHUR and MOHAN RAM, 1978). Such changes are used as cues by pollinators in deciding whether to visit or ignore the flower. In *Lantana*, thrips (MATHUR and MOHAN RAM, 1986) and butterflies (WEISS, 1991) forage on yellow flowers but not on red ones devoid of nectar. Fragrance is the other most important floral attractant. Flower scents are largely volatile derivatives of alcohols, esters, aldehydes and ketones. Some floral scents are distinctive and some are mimetic with smells of dung or mammal musk or insect pheromones. Many studies have clearly shown that pollen grains emit odours that differ from those of other flower parts and the pollen of other species. Pollenkitt is the main source of volatile substances and plays a role in guiding pollen-foraging insects to flowers (DOBSON, 1988). Insects are able to discriminate between odours of different pollen. Pollen odour is used by pollen-foraging insects not only to discriminate between plant species, but also to assess availability of pollen between individual flowers, this allows the pollinator to restrict its visit to the rewarding flower (DOBSON *et al*., 1996).

Floral rewards cater to an essential need of the pollinator to ensure repeated visitation. Pollen and nectar are the main nutritive rewards. Pollen is highly nutritious with around 25% carbohydrates, 25% proteins, 10% amino acids and 5% lipids. It is also rich in vitamins and minerals (SCHMIDT and BUCHMANN, 1992). Pollen grains of zoophilous species are generally ornamented with an oily coating. In some species they are held in clumps by viscin threads. Starch and oil are the major calorific reserves of pollen; smaller pollen tends to have oil and larger pollen starch. Pollen rich in starch tends to have a lower lipid content and is of lesser value for insects as a food source (BAKER and BAKER, 1979). Plants which offer pollen as the main or only source of energy tend to have oil-rich pollen. Bees gather pollen in special parts on their bodies, the pollen baskets. The pollen baskets of honey bees and bumble bees are on the hind legs, but on leaf cutting bees are under the abdomen. The insects carry the harvested pollen to their nests (SHIVANNA, 2003).

Nectar is largely a sugary solution and includes a minor proportion of amino acids, organic acids and minerals. Nectar is the main fuel for movement of anthophilous animals. Sucrose, glucose and fructose are the major sugars of nectar. The volume of nectar and concentration of sugars, sugar ratio and amino acid content are relevant for pollinators. The rewards are not confined only to nutrients. Non-nutritive rewards include nest materials, shelter and warm resting places, sexual attractants, mating and ovipositing sites (PATEL *et al*., 1993; KATHURIA *et al*, 1995).

2.1.6 Pollination postulates

To confirm the role of a vector in pollination, the following given postulates need to be demonstrated (COX and KNOX, 1988):

- Pollen transfer from anther to vector;
- Pollen transport by vector;
- Pollen transfer from vector to stigma;
- Fertilization from vector-deposited pollen;

- Flower advertisements perceived and used by pollinators;
- Flower reward consumed/used by vector as an integral part of the pollination process;
- Relative contribution of pollen and ovules to the next generation as a result of pollination; and
- Interrelationships between different vectors involved in pollination.

Effective techniques for demonstrating the above postulates are detailed in DAFNI (1992) and KEARNS and INOUYE (1993).

2.1.7 Pollen limitation and supplemental hand pollination

The production of seeds is frequently limited by pollen quantity or quality. When plants are not pollen limited, seed and fruit production are commonly limited by resource availability (PRIMACK and HALL, 1990; CALVO, 1993; EHRLÉN and ERIKSSON, 1995). Hence, most populations probably shift between reproductive effort being limited by pollen and resource availability over time. Inadequate pollinator service would favor the evolution of autonomous seed production by natural selection (DARWIN, 1876). Investigations have been done to examine the effects of natural variation in pollinator availability on pollen limitation of seed set (HERRERA *et al*., 2001; MOELLER and GEBER, 2005). Empirical attempts to test pollinator limitation must demonstrate either that (1) patterns of reduced pollinator visitation correlate with selection for self-fertilization in nature (FAUSTO *et al*., 2001); or (2) individuals lacking the ability to autonomously self-fertilize produce fewer seeds (KLIPS and SNOW, 1997; DAVIS and DELPH, 2005).

2.1.8 Pollination efficiency and pollinator effectiveness

The term pollination efficiency has been used ambiguously by pollination biologist (DAFNI, 1992; INOUYE *et al*., 1994; DAVIS, 1997). Understanding of each pollinator taxon’s effectiveness, visitation rate and variation in visitation rate over time is

essential to understand plant reproduction and floral evolution in generalist plant species. Pollination biologists have long recognized the need to estimate a relative value for each visiting taxon (JOHNSON and STEINER, 2000). Many studies evaluate pollination efficiency on the basis of quantitative aspects of pollination events, such as number of pollen grains deposted per stigma as a fraction of the number of pollen grains removed (SNOW and ROUBIK, 1987), and proportion of stigmas touched per insect visit (ROBINSON, 1979). Many other studies consider pollination efficiency on the basis of percent fruit- and seed-set as a consequence of pollination. KANDORI, (2002) defined pollinator importance as the total number of seed set by each pollinating taxon relative to the total number of seeds produced. Pollinator effectiveness can be estimated by many different methods such as the amount of removal and/or deposition of pollen (HERRERA, 1987; IVEY *et al.,* 2003), the pollen load on pollinators (TALAVERA *et al*., 2001; MOELLER and GEBER, 2005), and the probability of contacting stigma and anthers (LINDSEY, 1984). Combining estimates of pollen removal with pollen deposition effectiveness (or with seed set) can provide an estimate of pollinator efficiency, defined as the number of pollen grains deposited or set per pollen grain removed (YOUNG and STANTON, 1990).

2.1.9 Inbreeding depression

Inbreeding depression is the reduction in fitness of inbred versus outcrossed progeny caused by the expression of deleterious recessive alleles or the loss of heterozygote advantage (CHARLESWORTH and CHARLESWORTH, 1987). Understanding inbreeding depression and its fitness consequences remains an important and challenging topic in evolutionary biology because of its potential importance in the evolution of mating systems, life history traits, and its implications for conservation and plant breeding (MUSTAJÄRVI, 2005). Inbreeding depression may be caused by the expression of deleterious recessive alleles or over dominant loci, or both of these factors in combination (JOHNSTON and SCHOEN, 1995; CARR and DUDASH, 2003). In a population going through generations of inbreeding, deleterious recessive alleles responsible for inbreeding depression are exposed to selection as homozygotes and, therefore, may be purged (LANDE and SCHEMSKE, 1985). Inbred and outbred species may differ in the timing of inbreeding depression

(HUSBAND and SCHEMSKE, 1996). Outcrossers have strong inbreeding depression during early stages of their life-cycle, while selfers have milder inbreeding depression in later life stages. (HUSBAND and SCHEMSKE, 1996). Traits expressed in later life stages are often under polygenic control, and inbreeding depression in these traits is largely caused by mildly deleterious alleles. Several authors have reported reduction in inbreeding depression in inbred populations (BARRETT and CHARLESWORTH, 1991; DOLE and RITLAND, 1993; JOHNSTON and SCHOEN, 1996) or species (HUSBAND and SCHEMSKE, 1996; GOODWILLIE, 2000). The negative effects of inbreeding have since been observed in both outcrossing and selfing species for a variety of traits with consequences for offspring fitness (CHARLESWORTH and CHARLESWORTH 1987; KELLER and WALLER 2002). Examples of traits shown to be subjects to inbreeding depression include pollen quantity, number of ovules, amount of seed, germination rate, growth rate and competitive ability (KELLER and WALLER 2002).

2.1.10 Honey bees as pollinators

Honey bees (*Apis mellifera*, family Apidae), a social species native to Europe, the Middle East, and Africa, are the most important pollinating bee in the temperate developed world. They form large perennial colonies in hollow trees or other cavities, and they readily accept artificial hives. Man has cultured honey bees for thousands of years, and currently bee keeping practices are well known. Altogether, there are an estimated 60 million hives of *A. mellifera* managed by beekeepers around the world (DELAPLANE and MAYER, 2000). Honey bees are generalists that visit a wide assortment of blooming plants during a season. They are manageable, movable, well-known and effective pollinators for many crops, hence they are the standard against which all other bee pollinators are measured. However, because they are generalists, honey bees are not the best pollinator for every crop. Unlike some solitary bees whose life cycles and behaviours are perfectly matched for a particular crop, honey bees play the field for the richest reward. Thus, they are sometimes inefficient pollinators or essentially lured away if competing flowers are more attractive than the crop of interest (DELAPLANE and MAYER, 2000). Nevertheless, however, honey bees are not in the native range of *J. curcas*.

2.1.11 The potential role of pollination in crop improvement

Crop pollination is perhaps the most interdisciplinary field of study in all the agricultural sciences. It involves botany, entomology, plant breeding, horticulture, agronomy, genetics, bee breeding, ecology, agricultural economics, and pheromone biology. For many crops there is still paucity of information with which one can make good management decisions (DELAPLANE and MAYER, 2000). Fruits and seeds are the economic products of most crop plants. Effective pollination is a prerequisite for fruit- and seed-set. Therefore, successful pollination is of vital importance to realize optimal yield. In self-incompatible species, pollination is largely dependent on adequate cross-pollination. Even in self-compatible species, pollination is largely dependent on pollinating agents as automatic selfing seldom occurs or is insufficient in most of the self-compatible species. The majority of the crop plants, except cereals, are pollinated by insects, particularly bees. Adequate pollination is often a major constraint in many crop species due one or more of the following reasons (SHIVANNA, 2003):

- Drastic reduction in native pollinator populations because of the steady disappearance of natural habitats of insects, a marked increase in levels of pollutants and extensive use of environment-unfriendly chemicals, in particular herbicides and pesticides;
- Lack of a sufficient number of native pollinators due to the enormous increase in area covered by the same crop species (monoculture cropping); and
- Absence of natural pollinators for crops introduced from other regions.

Production in many of the fruit, seed and nut crops could be increased substantially by careful management of pollination (ROUBIK, 1995). Increased pollination efficiency can lead to an increase in crop value by increasing crop yield, uniformity, quality and decreasing the time of crop maturity. FREE (1993) and CURRIE (1997) reported several effective approaches to overcome pollination constraints. Some of these approaches are:

2.1.11.1 Habitat management

Increasing local populations of native pollinator species through habitat management. This is particularly useful when availability of nest sites is a limiting factor. Habitat management can be achieved by maintaining uncultivated strips along field margins and providing permanent nest boxes (POMEROY, 1981). Such strategies should also ensure availability of forage sources when the target crop is not in bloom. This approach also requires management of cropping practices in such way that the flowering period of the target crop coincides with the peak populations of the pollinator (FREE, 1993). However, management of habitat is more expensive, particularly in areas of intensive agronomic practices (TORCHIO, 1990).

2.1.11.2 Use of commercially managed pollinators

The most economically viable and effective approach to overcome pollination constraints has been use of commercially managed pollinators, in particular honeybees (*Apis s*pp), for pollination services. Honeybees are the most effective pollinators in a range of crop species (ROBINSON, 1979). Management of honey bees is convenient because of their large foraging populations, year-round availability and easy transportation (FREE, 1993). Increasing pollination efficiency through management of pollinators is warranted only when crops are pollen-limited and the cost involved is lower than the value realized through increases in crop production (CURRIE, 1997).

2.1.11.3 Spraying pollinator attractants on target crop

A number of studies have shown the potential of sprays with various substance on the target crop to attract pollinators. Sprays with dilute solutions of pheromones have shown considerable potential. Secretions from the Nasnov gland located on the dorsal side of the abdomen of worker bees consist of seven terenoids. Of these, geraniol and citral increase honey-bee foraging activity. Similarly, the queen honey-bee secretes a five component pheromone from its madibular glands. Sprays of

synthetic mandibular pheromones do increase honey-bee foraging activity and crop yield under a wide range of conditions (CURRIE *et al.*, 1992; WINSTON and SLESSOR, 1993). Application of pheromones seems to be particularly effective on crops with flowers relatively unattractive to bees or during inclement weather conditions (SHIVANNA, 2003). Apart from pheromones, sprays of synthetic plant volatiles isolated from nectar or pollen are also effective in attracting honey-bees (DOBSON, 1994). Sprays containg food supplement such as Beeline® have also been reported to act as bee attractant in some crop species (MARGALITH *et al*., 1984).

2.1.11.4 Introduction of pollinators

Introduction of pollinators is one of effective approaches when crops are grown in areas where natural pollinators are absent, as often happens when a crop is introduced from one country to another. This approach involves detailed studies on the biology of the pollinator and monitoring its establishment in the new area (SHIVANNA, 2003). Oil palm (*Elaeis guineesis*) is native to Africa and Central South America. It was introduced to Malaysia and Indonesia and is grown extensively. In its native habitat, oil palm is pollinated by wind as well as many insects, in particular weevils. In many parts of Malaysia, where pollinating insects are absent, natural pollination was inadequate. Introduction of the weevil *Elaeidobius kamerunicus*, an important pollinator of oil palm from Cameroon is a successful example of such an approach. Introduction of the weevils has markedly increased the yield. Over the first seven months after weevil introduction, oil yield increased 20-53% (SYED, 1979).

2.1.11.5 Supplementary pollination

Assisted/supplementary pollination through pollen sprays or other methods such as manual hand pollination is the most effective technique for sustaining crop yield (WILLIAMS and LEGGE, 1979; HOPPING and JERRAM, 1980b). This is routinely carried out for small-scale production of a few crops, such as passion-fruit (ROUBIK, 1995). In high value plantation crops such as oil palm, pollination is a major constraint even in the presence of weevils, especially in younger plantations.

Although oil palm is monoecious, the male and female phases alternate, each extending for many months, thus at any given time the plant will be either male or female phase dominated. Insufficient number of plants in the male phase in the plantation and unfavourable weather conditions reduce pollination efficiency. Assisted pollination is a common practice in oil-palm plantations particularly in younger plantations. Assisted pollination has been reported to increase yield 20-150%, depending on age of the plants and weather conditions (SHIVANNA, 2003). Assisted pollination requires standardization of protocols for pollen collection. Different methods have been assayed for assisting pollination in oil palm. Hand pollination is regularly practiced for the *Vanilla* orchid. This plant is a native of southern Mexico and Central America where it is pollinated by the euglossine bee *Eulaema* (ROUBIK, 1995). *Vanilla* is grown extensively in many parts of tropical Asia where the pollinator is absent, hand pollination is routinely carried out to induce fruit-set (SHIVANNA, 2003).

Floral biology and pollination ecology of *J. curcas* were studied by RAJU and EZRADANAM (2002); BHATTACHARYA *et al.* (2005); CHANG-WEI *et al.* (2007). However, these studies did not cover the implementation of pollination management for yield improvement in this plant. Therefore, **Table 2.8** summarieze some pollination improvement done on other crop species.

Table 2.8 Improvement done on different crop species by pollination management.

Crop	Aspects	Reference
Avocado (*Persea americana*)	Pollinators; pollination requirements pollinator behaviour; and self- and cross-pollination	VITHANAGE (1990); ISH-AM *et al*. (1999); DELAPLANE and MAYER (2000); DEGANI *et al*. (2003).
Apple (*Malus domestica*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000).
Almond (*Prunus dulcis*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000).
Cotton (*Gossipium hirsutum*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000).
Cranberry (*Vaccinium macrocarpon*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000).
Kiwifruit (*Actinidia deliciosa*)	Pollinators and pollination requirements; influence of honey bee on pollination and fruit quality	DELAPLANE and MAYER (2000); HOWPAGE and SPOONER-HART (2001).
Peach and nectarine (*Prunus persica*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000)
Pear (*Pyrus communis*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000)
Plum and prune (*Prunus domestica*)	Pollinators and pollination requirements	DELAPLANE and MAYER (2000)
Raspberry (*Rubus idaeus*	Pollinators and pollination requirements	DELAPLANE and MAYER (2000)
Guava (*Psidium guajava* L.)	Visitation effectiveness by honey bees	FREITAS and ALVES (2008)
Mango (*Mangifera indica*)	Pollinators	DAG and GAZIT (2000)
Coffee (*Coffea canephora*)	Pollinator effectiveness	ROUBIK (2002); KLEIN *et al*. (2003).
Sunflower (*Helianthus annuus*)	Pollinators effectivness and pollination requirements	DELAPLANE and MAYER (2000); NDERITU *et al*. (2008).

2.2 POLLEN BIOLOGY

Pollen grains embody the male partners in sexual reproduction. They are generally shed in a desiccated condition and their moisture level is less than 20%. At the time of shedding, pollen grains are either two-celled – a large vegetative cell enclosing a generative cell; or three-celled – a vegetative cell and two sperm cells formed by the division of the generative cell. There is considerable variation in the shape and size of pollen grains (SHIVANNA and SAWHNEY, 1997). The wall of the pollen grain is made up of two layers: an outer, acetolysis-resistant exine composed of sporopollenin and an inner pectocellulosic intine. One of the conspicuous structural features of pollen grains is the ornamentation of the wall formed by the outer part of the exine (CRESTI *et al*., 1992). Pollen biology involves a comprehensive understanding of the structural and functional aspects of pollen grains. The main function of the pollen is to discharge male gamets in the embryo sac for fertilization and for subsequent seed development. This function depends on the successful completion of a number of sequential events. The following are considered the major events in pollen biology (SHIVANNA and SAWHNEY, 1997).

- Pollen development;
- Free dispersed phase;
- Pollination; and
- Fertilization.

2.2.1 Pollen development

Pollen grains develope inside the anther and are dispersed by dehiscence of the anther. After dispersal, pollen grains remain as independent functional units and are exposed to the prevailing environmental conditions for varying periods. Depending on the period and severity of the environment, the quality of the pollen grains, particularly their viability and vigour, may be affected during this pollination phase. Eventually pollen grains are deposited on the stigma (pollination) through biotic or abiotic agents (SHIVANNA and SAWHNEY, 1997). Structural details of pollen

development are quite uniform in most of the species studied. The main structural events associated with pollen development are:

(i) The formation of a syncytium of microspore mother cells (MMCs), also referred to as pollen mother cells (PMCs) or meiocytes, in each anther locule, followed by the isolation of each MMC and the resulting microspores encased in a callose wall;

(ii) Cytoplasmic reorganization resulting in the breakdown of most of the RNA and ribosomes of MMCs, and differentiation of plastids and mitochondria;

(iii) Release of miocrospores by the activation of callase;

(iv) Development of microspores by the synthesis and build up of RNA, ribosomes, and proteins, and redifferentiation of plastids and mitochondria;

(v) Asymmetric division of the microspore; and

(vi) Desiccation and dispersal of pollen grains.

2.2.2 Structure of the pistil

2.2.2.1 The stigma

Based on the presence or the absence of stigmatic exudates on its surface at the time of pollination the stigma can be one of two types: (1) a wet stigma or (2) a dry stigma. Each of these types is further divided on the basis of the presence or absence of papillae on the receptive surface and anatomical details. Irrespective of the morphology, the stigma invariability contains extracellular components of the receptive surface (HESLOP-HARRISON and SHIVANNA, 1977). These components are highly heterogenous and include lipids, proteins, and glycoproteins, different carbohydrates, amino acids, and phenols. A range of enzymes have also been localized; the non-specific esterases are the predominant ones, cytochemical demonstration of non-specific esterases has become a standard method of

localization of the receptive surface of the stigma (SHIVANNA and SAWHNEY, 1997).

2.2.2.2 The style

There are two types of style: (1) the solid or (2) the hollow (SHIVANNA and SAWHNEY, 1997). In the solid style, a core of transmitting tissue, starting from the secretory tissue of the stigma, traverses the whole length of the style. The transmiting tissue is made up of elongated cells connected end to end through the plasmodesmata (SANDERS and LORD, 1992). The intercellular substance is composed predominantly of pectin but it also contains proteins, glycoproteins, and often lipids; it also respond to many enzymes, such as esterases, acid phosphatase and peroxidases. A number of transmitting tissue-specific, proline-rich proteins have been localized in the intercellular matrix (WANG *et al*., 1993). The cells of the transmitting tissue exhibit normal ultrastructural profiles with numerous mitochondria, active dictyosomes, rough endoplasmic reticulum (RER), plastids, and ribosomes. Endoplasmic reticulum (ER) and Golgi vesicles have been implicated in the secretion of an intercellular matrix (KRISTEN *et al*., 1979). Irrespective of the structural diversity of the stigma and style, the surface of the stigma and the path of pollen tube growth in the pistil invariably contain extracellular components, which come into direct contact with the pollen grain and pollen tube (MIKI-HIROSIGE *et al.*, 1987).

2.2.2.3 Ovary and ovule

The transmitting tissue/canal cells of the style continue into the ovary as the placenta. The ovule, the seat of female gametophyte (embryo sac), develops on the placenta. Extensive studies have been carried out on the structural details of the ovule and embryo sac (CRESTI *et al*., 1992; GASSER and ROBINSON-BEERS, 1993; RUSSELL, 1993).

2.2.3 Pollen-pistil interaction

Pollen grains are deposited on the stigma either by close proximity of the anthers or by biotic/abiotic agents. Successful pollination initiates as a number of sequential events that culminate in the discharge of male gametes into the embryo sac. All these events, from pollination to the release of male gametes in the embryo sac, are included in pollen-pistil interaction (SHIVANNA and SAWHNEY, 1997). Pollen-pistil interaction involves a series of “dialogues” between the male gametophyte and sporophytic tissues of the stigma and style. These interactions result in generation of appropriate signals which elicit the required responses in the pollen and/or pistil. Successful completion of pollen-pistil interaction is an essential requirement for fertilization and seed-set. Any deviation of these sequential events prevents fertilization and consequently fruit- and seed-set. Recently, there has been an increasing realization of the need for understanding the details of pollen-pistil interaction for effective manipulation in crop production and crop improvement (SHIVANNA, 2003). This realization has resulted in extensive studies on the structural and functional aspects of pollen-pistil interaction. Pollen-pistil interaction has been covered by many reviews (KNOX *et al*., 1986; CRESTI *et al*., 1992; SHIVANNA and SAWHNEY, 1997; DE GRAAF *et al*., 2001). The ability of the gamete to establish recognition so as to facilitate fusion of only the right type of gametes is the prerequisite for sexual reproduction in any organism. In seed plants pollen grains act as vehicles for gamete transmission. In gymnosperms pollen grains are deposited in the pollen chamber and a short pollen tube is produced before the male gametes are released (OWENS *et al*., 1998). In zooidogamous gymnosperms (*Cycads* and *Ginkgo*) pollen tubes are not directly involved in the transfer of sperm to the egg; they function as haustorial organs to acquire nutrition from the surrounding sporophytic tissues of the ovule (JOHRI, 1992).

2.2.4 Pollen viability

Pollen viability refers to the ability of the pollen to perform its function of delivering male gametes to the embryo sac. The period for which pollen grains remain viable varies greatly from species to species. On the basis of their longevity pollen grains of

different species can be grouped into three categories (BARNABAS and KOVACS, 1997).

- Short-lived pollen: pollen grains lose their viability within a few days (*Cyperaceae, Juncaceae*). In some species viability is lost in less than an hour (sorghum, wheat) (LANSAC *et al*., 1994);
- Pollen with medium life span: pollen of majority of families, such as Solanaceae, Liliaceae and Amaryliaceae, fall within these extremes; they maintain viability for 1-3 months; and
- Long-lived pollen: pollen of many Gymnosperms (Pinaceae and Gingkoaceae) and members of several angiosperm families, such as Leguminaceae, Rosaceae and Arecaceae, maintain viability for over 6 months.

2.2.4.1 Tests for viability

Assessment of pollen viability is important for studies on pollen storage, reproductive biology and hybridization (DAFNI and FIRMAGE, 2000).

2.2.4.1.1 Fruit- and seed-set

As viability refers to the ability of pollen to deliver functional gametes to the embryo sac, the most authentic test for viability would be to assess the fertilization capacity of the pollen sample as measured by fruit- and seed-set following controlled pollination (SHIVANNA, 2003). However, this test has many limitations for use as a routine test: (i) it is laborious and time-consuming; (ii) many other factors such as stigma receptivity and incompatibility have to be taken into consideration to perform this test; (iii) seed-set is not an inevitable outcome of fertilization as many post-fertilization factors associated with seed development may influence seed-set; (iv) this test cannot be used in apomictic species; (v) it can be used only during the flowering period of the species; (vi) it can be used more as a qualitative than quantitative test, particulary in systems with fewer ovules, as germination of a limited number of pollen is enough to induce full seed-set. Therefore, assessment of pollen viability through fruit- and seed-set is not practicable as a routine test, although it can be used to confirm the results of other tests (SHIVANNA, 2003).

2.2.4.1.2 Pollen germination and pollen tube growth in the pistil

Some attempts have been made to assess pollen viability by studying pollen germination and pollen tube growth in the pistil following controlled pollination. In *Brassica oleraceae*, for example, pollen samples which produce about 70 pollen tubes in the style are considered fully viable (OCKENDON, 1974). Although this method markedly reduces the time taken compared to the fruit- and seed-set method, it has the most of the other limitations associated with fruit- and seed-set. Also, it is not always feasible to quantify the number of pollen tubes growing in the style.

2.2.4.1.3 Non – vital stains and other tests of limited use

Many alternative methods which are simple, convenient and rapid have been developed. Many of the staining tests using non-vital stains such as iodine in potassium iodide, alanine blue in lactophenol, acetocarmine, acid fuchsin and Alexander's stain (ALEXANDER, 1980), essentially assess the presence of contents in the pollen. They are satisfactory in assessing pollen sterility but are not dependable for testing viability (HESLOP-HARRISON *et al*., 1984). Some non-permeating satains, such as Evans blue and phenosafranin (WIDHOLM, 1972) which do not enter plasma membranes of living cells but stain the cytoplasm of dead cells are reportedly suitable for assessing pollen viability. Inorganic acid tests based on bursting of viable pollen in inorganic acids and formation of instant pollen tubes from hydrated pollen, although very simple and rapid, are largely not used because data for establishing their correlation with true viability are lacking. A cytochemical test, termed the benaidine test, which is based on the oxidation of benzidine by peroxidase in the presence of hydrogen peroxide, has been used for assessing viability of pollen of many species. This test, however, is not often used because of benzidine toxicity and also the availability of better and more effective tests (SHIVANNA, 2003).

2.2.4.1.4 Tetrazolium test

This test is based on reduction of soluble colourless tetrazolium salt to reddish insoluble formazan in the presence of dehydrogenase. Following incubation of pollen grains in tetrazolium solution for 30-60 min, pollen grains which take a reddish colour are scored as viable. The most commonly used salt is TTC (2,3,5-triphenyl tetrazolium chloride). Satisfactory results were reported when using this test in assessing pollen viability in several species (COLLINS *et al*., 1973). Another tetrazolium salt is 2,5-diphenyl tetrazolium bromide (MTT or thiazolyl blue). This test detects the presence of dehydrogenase. The test solution consists of a 1% concentration of the substrate MTT or thiazolyl blue (2,5-diphenyl tetrazolium bromide) in 5% sucrose. The pollen grain is considered viable if it turns deep pink or if it presented no colour but showed irregular black lines over its surface (KHATUN and FLOWERS 1995, DAFNI and FIRMAGE, 2000) . Often, results of the tetrazolium test did not correlate with seed-set data (BARROW, 1983) or the *in vitro* germination test (HESLOP-HARRISON *et al*., 1984). Another limitation is that correlation of responding pollen shows a gradation from very light to dark red with the result that the cut-off point for scoring viable pollen becomes subjective.

2.2.4.1.5 *In vitro* germination test

This test is the most commonly used and acceptable for assessing pollen viability. It is rapid, simple and the results of *in vitro* germination generally correlate with seed set data (JANSSEN and HERMSEN, 1980). However, this correlation depends on optimization of the medium and other cultural conditions to induce germinability in most of the viable pollen. In a suboptimal medium, this test gives negative results. Furthermore, many of the stored pollen samples which fail to germinate *in vitro* are often found to be capable of inducing fruit- and seed-set following pollination. A major limitation of this test is lack of optimal germination medium for pollen of many species, particularly the 3-celled pollen ones (SHIVANNA, 2003).

2.2.4.1.6 Aniline blue in lactophenol

Aniline blue in lactophenol stains callose, and provides (at best) only rough estimates of viability (STANLEY and LINSKENS, 1974; HESLOP-HARRISON *et al*., 1984). Aniline blue in lactophenol stains non-abortive pollen but not abortive pollen. MAYER (1991) used this stain to evaluate male fertility of *Wikstroemia* (Thymelaeaceae) hybrids by scoring a minimum of 300 grains per sample. Darkly stained grains were considered viable. The accuracy of this method was confirmed with a nitro blue tetrazolium stain for dehydrogenase activity.

2.2.4.1.7 Fluorescein diacetate test (FDA)

The flurescein diacetate (FDA) test, often referred to as the fluorochromatic reaction (FCR) test, was introduced by HESLOP-HARRISON and HESLOP-HARRISON (1970) as a test for pollen viability. The FDA assesses two properties of the pollen: (i) integrity of the plasmamembrane of the vegetative cell and (ii) presence of active esterases in the pollen cytoplasm. Non-polar, non-fluorescing FDA passes freely through the pollen membrane and enters the pollen cytoplasm. Hydrolysis of FDA by the activity of esterases results in fluorescein which is fluorescent. Since the fluorescein does not pass through the intact plasmamembranes as readily as FDA, it accumulates in the pollen cytoplasm. Such pollen grains show bright fluorescence under a microscope. Pollen grains that do not have intact plasmamembrane allow fluorescein to move out readily and thus result in uniform background fluorescence. Likewise, if there are no active esterases in the pollen cytoplasm, fluorescein is not formed and hence pollen grains do not show fluorescence (SHIVANNA, 2003). The FDA test has proven satisfactory in assessing pollen viability in a number of species (SEDGLEY and HARBARD, 1993). It reportedly has wider applicability and better resolution than other prevailing tests for assessing pollen viability of cotton (SHIVANNA, 2003). In species in which the medium used for *in vitro* germination of pollen is optimal, a close correlation exists between the FDA test and the *in vitro* germination test (HESLOP-HARRISON *et al*., 1984). In the absence of optimal medium, the FDA test gives a better index of viability than *in vitro* germination (SHIVANNA *et al.,* 1991a).

2.2.5 Pollen vigour

Pollen vigour refers to the speed of germination and the rate of pollen tube growth. It differs from viability since viable pollen samples may show differences in vigour (SHIVANNA, 2003). As seeds and pollen grains are very similar in many physiological manifestations, it was suggested that pollen grains also exhibit reduction in vigour before loss of viability (SHIVANNA and CRESTI, 1989; SHIVANNA *et al*., 1991a). It was reported that ageing, and many environmental stresses, in particular desiccation, temperature and humidity affect pollen vigour before affecting viability (SHIVANNA *et al*., 1991a,b).

2.2.5.1 Tests for vigour

2.2.5.1.1 *In vitro* germination

Apart from its use in assessing pollen viability *in vitro* germination can also be used to assess pollen vigour (SHIVANNA and CRESTI, 1989). In viability tests using *in vitro* germination, the capacity of pollen to germinate is assessed without consideration of the time factor; the cultures are generally scored after maintaining them for a much longer period than that required for germination. To assess the vigour, however, germination is scored at intervals over a period of time and compared with the values obtained for control pollen (fresh pollen). Pollen grains with reduced vigour took a longer time to attain maximum germinability than did fresh pollen.

2.2.5.1.2 *Semivivo* technique

In the *semivivo* technique, the pollen sample to be tested is used to carry out controlled pollination. The pistil used for pollination can either be maintained on the plant or be excised and maintained in the laboratory (SHIVANNA, 2003). Pollinated pistils are maintained (for 3-6 h) until pollen grains germinate on the stigma and pollen tubes grow down for some length in the style. After a suitable time of incubation, the style is cut ahead of the growing pollen tubes and the cut end of the style is implanted in the agar medium containing the components of the pollen germination medium. The pollen tubes continue their growth and enter the agar

medium through the cut end of the style (SHIVANNA *et al.,* 1991a). The number of pollen tubes that emerge into the medium are counted and their length measured either *in situ* or after pulling out the implant and observing under a microscope. The *semivivo* technique requires some preliminary studies on pollen germination and pollen tube growth *in vivo.* Pollen vigour is assessed on the basis of the time taken for pollen tube emergence into the medium and the number of emerged pollen tubes.

2.2.5.1.3 *In vivo* pollen germination and pollen tube growth

Pollen vigour can be assessed by studying pollen germination and pollen tube growth at regular intervals in pistils pollinated with pollen samples. Comparison of the extent of pollen germination and pollen tube growth in pistils pollinated with the test pollen sample and those with fresh pollen indicate the vigour of the pollen sample. Another simple method for assessing pollen vigour is to excise the stigma and a part of the style at different intervals after pollination (SHIVANNA, 2003). Pollen tubes, which grow through the excision zone before excision, continue to grow and affect fertilization (JAUH and LORD, 1995). Thus more vigorous tubes would grow through the excision zone earlier and result in seed-set while less vigorous pollen tubes may not grow or only a few may grow through the excision zone and this would result in no seed-set or reduced seed-set.

2.2.6 *In vitro* pollen germination and pollen tube growth

Germination of pollen is the first morphogenetic event in fulfilling its function of transport and discharge of sperm cells into the embryo sac. *In vivo* studies on pollen germination are difficult to perform due to involvement of pistillate tissue. Since pollen grains of a large number of species readily germinate *in vitro* on a simple medium, *in vitro* germination has been extensively used in studies on structural and physiological details of germination and tube growth. Two-celled pollen grains in general are more amenable to *in vitro* germination compared with three-celled pollen. Details of the processes involved in pollen germination and pollen tube growth are discussed in many reviews (HESLOP-HARRISON, 1988; STEER and STEER, 1989; MASCARENHAS, 1993; DERKSEN, 1996).

2.2.6.1 Germination requirements

2.2.6.1.1 Hydration

As pollen grains are shed under desiccated conditions, hydration is a basic prerequisite for germination. In liquid medium hydration is rapid and completed within a few minutes. In semi-solid medium, it is slower and rate of hydration depends on moisture level of the medium. Many studies have shown that the rate of hydration is critical for optimal germination, particularly for desiccated pollen (SHIVANNA, 2003). Controlled hydration seems to provide better conditions for restoration of membrane integrity and thus prevents leakage of metabolites when transferred to the culture medium (SHIVANNA and HESLOP-HARRISON, 1981). GILLISSEN (1977) reported that in *Petunia* controlled hydration affects rigidity of the pollen wall. Pollen grains exposed to controlled hydration before culture showed a 3-fold increase in volume after culture compared to direct culture which showed only a 2-fold increase.

2.2.6.1.2 Carbohydrate source

A suitable carbohydrate source in the medium is required for adequate pollen germination and tube growth. A carbohydrate source serves two functions: (i) It maintains the required osmotic potential of the medium, and (ii) serves as substrate for pollen metabolism (NYGAARD, 1977). The former is perhaps more important than the latter. In short-term cultures pollen grains contain sufficient endogenous sugars for germination and early tube growth. In long-term cultures, exogenous carbohydrates are needed for continued tube growth. Sucrose has been the most commonly used carbohydrate source. In some species other sugars such as glucose, fructose and raffinose also support pollen germination and tube growth. In general, 2-celled pollen require a lower sucrose level (10-15%), while 3-celled pollen require higher levels (>20%).

2.2.6.1.3 Boron

Boron is a regular component of all pollen germination media. Boric acid is generally used as the boron source. In the absence of boron, pollen grains generally show poor germination and a high degree of bursting. Pollen grains are believed to be deficient in boron which is compensated by high levels of boron in the stigma (SHIVANNA, 2003). Boron seems to affect various pathways of carbohydrate metabolism. A major metabolic activity of germinated pollen is the synthesis of pollen wall components to cope with the requirement for long pollen tubes needed to grow through the length of the style to reach the embryo sac. It is suggested that boron stimulates conversion of myo-inositol, to wall polysaccharides, thus enabling the enzyme to bind to inositol or inositol derivatives (MAITI and LOEWUS, 1978). Boron also seems to affect the avilabilty of substrates for other pathways of carbohydrate metabolism (BIRNBAUM *et al*., 1977).

2.2.6.1.4 Calcium

Calcium is another important inorganic requirement for pollen germination and pollen tube growth. The amount of calcium present in the pollen is generally far less than that present in the vegetative parts and seeds (MCLELLAN, 1977). In some species calcium is not required for pollen germination and this is explained on the basis that pollen grains of such species contain higher levels of endogenous calcium. Many functions have been attributed to calcium. It has been suggested that calcium gives rigidity to the pollen tube wall by binding pectic carboxyl groups (KWACK, 1967). Calcium also has a role in controlling permeability of the pollen membrane. The most important role of calcium has been shown to be in regulating tip growth of the pollen tube.

2.2.7 Phases of germination and tube growth

2.2.7.1 Lag phase

The time between pollen hydration and pollen tube emergence is termed the lag phase and varies from species to species. In some species it is limited to a few minutes, while in others it extends from about 30 min to a few hours (SHIVANNA *et al.,* 1991a). A close correlation exists between duration of the lag phase and level of mitochondrial differentiation in the pollen at the time of dispersal. The differences in the lag phase correlates with differences in their requirements for protein synthesis for germination. Pollen grains with short lag phases do not require protein synthesis for germination, while those with a long lag phase do.

2.2.7.2 Pollen tube emergence

Pollen tubes emerge from the germ pores. In species with more than one pore, generally a single pollen tube emerges through one of them. Occasionally more than one tube arises from a pollen grain but only that one among them which receives the nuclei will continue growth; the other will abort. Germ pores are generally free from exine and thus do not offer much resistance to the emerging tubes (SHIVANNA, 2003). Following pollen tube emergence, a clear zonation is established at the growing tip (CRESTI *et al*., 1977).

2.2.7.3 Pollen tube growth

Pollen tube structure and mode of growth at the stigma is very similar to pollen tube growth *in vitro* (HERRERO and DICKINSON, 1979) since during this phase the pollen tubes grow autotrophically at the expense of the pollen grain reserves (HERRERO and DICKINSON, 1981). When the pollen tubes enter the style there is an acceleration of growth (HERRERO and DICKINSON, 1980) that is accompanied by a change from autotrophic to heterotrophic metabolism (HERRERO and DICKINSON, 1981). The style contains abundant starch reserves which disappear as the pollen tubes grow through the stylar tissue (HERRERO and DICKINSON, 1979).

When the pollen tubes reach the base of the style and enter the ovary they meet the obturator which is a placental protuberance connecting the style with the ovule micropyle. A histochemical study of this structure (ARBELOA and HERRERO, 1987a) reveals that when the pollen tubes arrive at the obturator they stop growing and growth is not resumed until five days later. On the arrival of the pollen tubes the obturator cells are full of starch reserves but five days later starch disappears from these cells and a secretion that stains for carbohydrates and proteins is produced. Concomitant with the production of this secretion growth of the pollen tubes is resumed on the obturator. The fact that pollen tubes appear to require this secretion to grow reinforces the idea that pollen tube growth along the pistil is heterotrophic (HERRERO and DICKINSON, 1979). However, a major difference exists between growth in the style and on the obturator. While in the transmitting tissue starch digestion is triggered by pollination and only occurs in compatible matings (HERRERO and DICKINSON, 1979). On the obturator this process is independent of pollination and appears to be a maturative stage of the pistil for it takes place in a similar way in pollinated and unpollinated flowers (ARBELOA and HERRERO, 1987a). Once the pollen tubes have passed along the obturator, callose starts to accumulate on this structure. This mechanism confers the obturator a critical role in controlling pollen tube penetration into the ovary since it acts as a bridge either connecting or isolating the ovary to the style. Thus, pollen tube growth is not possible before the secretion phase, neither is it possible later once the obturator degenerates. This mechanism, apart from having a role in pollen tube growth control, may play a significant part in preventing infection.

2.2.8 The role of pollen biotechnology in crop improvement

Pollen biotechnology is the management or manipulation of pollen grains for crop production and improvement. Pollen grains can be manipulated during all phases of pollen biology-development, free-dispersed phase, pollination, pollen pistil-interaction and fertilization. Integration of pollen biotechnology into the traditional breeding programme offers effective approaches in a number of areas of production and improvement of crops. Pollen biotechnology decreases the time and cost of crop improvement and increases its efficiency. SHIVANNA, (2003) demonstrated two of the potential areas of pollen biotechnology for effective application, which are:

(1) overcoming pollination constraints; and

(2) overcoming crossability barriers and many other constraints to transfer genes across species barriers.

2.3 PLANT HORMONES

2.3.1 Introduction

Plant hormones are a group of naturally occurring, organic substances which influence physiological processes at low concentrations. The processes influenced are mainly those of growth, differentiation and development. The effects produced by each hormone have been elucidated largely from exogenous applications. Plant hormones do not act alone but in conjunction, or in opposition, to each other such that the final condition of growth or development represents the net effect of a hormonal balance (DAVIES, 2004). Plant growth regulators (PGRs) are organic compounds other than nutrients (supplying either energy or mineral elements) that, in small amounts, promote, inhibit, or otherwise modify any physiological process in a plant. The term PGRs include both, naturally occurring plant growth substances, or phytohormones, as well as synthetic compounds or chemical analogs (BASARA, 2000). Plant growth regulators PGRs have been an important component in agricultural production even prior to the identification of plant hormones. PGRs are used on millions of hectares worldwide on a diversity of crops. Most of these applications are, however, confined to high-value horticultural crops rather than field crops, although there are significant exceptions. Significant opportunities exist for the development of plant growth regulators to increase yield in the major crops. PGRs are useful because they can in some way modify plant development. This may occur by interfering with biosynthesis, metabolism, or translocation of plant hormones, or the PGRs may replace or supplement the plant hormones when their endogenous levels are below that needed to change the course of plant development (GIANFAGNA, 1995). There are five well-established categories of classical phytohormones, namely, auxin, gibberellins, cytokinins, abscisic acid and ethylene. More recently several other compounds that can regulate various facets of plant

growth and development have been described, such as, oligosaccharins, brassinosteroids, jasmonates, salicylates, and polyamines (BASARA, 2000).

2.3.2 Auxin

2.3.2.1 Nature, site of biosynthesis and transport

Indole-3-acetic acid (IAA) is the main auxin in most plants. “Compounds which serve as IAA precursors may also have auxin activity, such as indoleacetaldehyde. Some plants contain other compounds that display weak auxin activity, such as phenylacetic acid (WIGHTMAN and LIGHTY, 1982). IAA may also be present as various conjugates, such as indole-3-acetyl-L-aspartate (IAAsp) (PARK and PARK, 1987)”. Several synthetic auxins are used in commercial applications (GIANFAGNA, 1995). IAA is synthesized from tryptophan, primarily in leaf primordia and young leaves, and in developing seeds. IAA transport is cell to cell. Transport to the root probably also involves the phloem (DAVIES, 1995).

2.3.2.2 Effects of auxins:

According to DAVIES, (1995) the general effects of auxins on the plants are:

- Stimulation of cell enlargement and stem growth;
- Stimulation of cell division in the cambium and, in combination with cytokinin, in tissue culture;
- Stimulation of differentiation of phloem and xylem;
- Stimulation of root initiation on stem cuttings, and also the development of branch roots and the differentiation of roots in tissue culture;
- Mediation of the tropic (bending) response of shoots and roots to gravity and light;
- Represses the growth of lateral buds when supplied to the apical bud;

- Delays leaf senescence and may inhibit or promote (via ethylene) leaf and fruit abscission depending on the timing and position of the source;
- Assimilate movement is enhanced towards an auxin source, possibly by an effect on phloem transport;
- Delays ripening;
- Promotes flowering in Bromeliads;
- Stimulates growth of flower parts; and
- Promotes femaleness in dioecious flowers (via ethylene).

2.3.2.3 Commercial uses of auxins in agriculture

Indole acetic acid (IAA) is not in itself useful in agriculture because it is rapidly broken down to inactive products by light and microorganisms. Nevertheless, a number of synthetic compounds were found to act similarly to IAA in the auxin bioassay tests. Indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) were found to increase root development in the propagation of stem cuttings. NAA and naphthalene acetamide (NAAm) are used to reduce the number of fruit that have set in apple, whereas 4-chlorophenoxyacetic acid (4-CPA) is used to increase fruit set in tomato. The auxin 2,4,5-trichlorophenoxpropionic acid (2,4,5-T) and the dichlorophenoxy analog (2,4-D) are used to prevent abscission of mature fruit in apple (DAVIES, 1995).

2.3.2.3.1 Propagation

Rooting of stem cuttings was one of the first uses of auxins. The most common compounds used are IBA, NAA and 2,4-D. The auxin stimulates root development by inducing root initials that differenciate from cells of young secondary phloem, cambium, and pith tissue (DAVIES, 1995).

2.3.2.3.2 Stimulation of fruit set

One of the first recorded effects of auxins was the stimulation of fruit set in unpollinated ovaries of Solanaceous plants. Pollen is a rich source of auxin, and in some species pollination alone is all that is required for fruit set to occur. In tomato, chemical stimulation of fruit set is all that is needed for fruit growth to take place. The compounds that block the transport of auxin from the ovary to the pedicel of the flower also stimulate fruit set. It seems likely, therefore, that under environmental conditions somewhat inhibitory to fruit set, application of auxin to flowers could promote this process (DAVIES, 1995).

2.3.2.3.3 Prevention of fruit drop and chemical thinning

Frequently, the mature fruit of apple, pear, lemon and grape fruit will abscise prior to the time of commercial harvest. This obviously reduces the potential crop yield, and may result in the tendency to begin harvesting the crop earlier than is desirable, resulting in lower quality fruit. Under natural conditions, there seems to be an inverse relationship between auxin content of the fruit, and the tendency toward abscission (LUCKWILL, 1953). In order to improve flower bud production and fruit size in apple, thinning should take place within 30 days from full bloom. The two auxin-type compounds used in chemical thinning of apple and pear are NAA and NAAm. LUCKWILL (1953) proposed that fruit abscission occurs because NAA and NAAm induce embryo abortion. Without seed growth, fruit senescence takes place prematurely. While the number of viable seeds is often correlated with fruit abscission, this is not always the case, suggesting that embryo abortion may not be a primary factor resulting in abscission. NAA does cause increased ethylene evolution from apple fruit within one day after application (WALSH *et al*., 1979). Ethylene is known to reduce auxin transport from leaf blade to petiole (BEYER, 1973), and to induce the synthesis of enzymes that degrade the abscission zone (ABELES *et al*., 1971).

2.3.3 Cytokinins (CKs)

2.3.3.1 Nature, site of biosynthesis and transport

CKs are adenine derivatives characterized by an ability to induce cell division in tissue culture. The most common cytokinin base in plants is zeatin. Cytokinins also occur as ribosides and ribotides. CK biosynthesis is through the biochemical modification of adenine. It occurs in root tips and developing seeds. CK transport is largely via the xylem from roots to shoots (MCGAW, 1995).

2.3.3.2 Effects of cytokinins

According to DAVIES, (1995) the general effects of cytokinins on plants are:

- Exogenous applications induce cell division in tissue culture in the presence of auxin;
- Promote shoot initiation in tissue culture and crown gall;
- Induce bud formation in moss;
- Applications, or the increase in its levels in transgenic plants with genes for enhanced CK synthesis, can cause the release of lateral buds from apical dominance;
- Leaf expansion resulting solely from cell enlargement. This is probably the mechanism by which the total leaf area is adjusted to compensate for the extent of root growth, as the amount of CKs reaching the shoot will reflect the extent of the root system;
- Delay leaf senescence;
- May enhance stomatal opening in some species; and
- The application leads to an accumulation of chlorophyll and promotes the conversion of etioplasts into chloroplasts.

2.3.3.3 Commercial uses of cytokinins in agriculture

Benzyladenine is used on white pine to increase lateral bud formation and subsequent growth and branching. Tetrapyranylbenzyladenine (Accel) is registered

for use on carnations and roses for increasing lateral branching. Promalin, a mixture of benzyleadenine and GA4/7 is used to control fruit shape in ‘Delicious’ apple. Pomalin applied at bloom will increase the length to diameter ratio of the fruit (WILLIAMS and STAHLY, 1969). Promalin is used to increase lateral branching in non-bearing apple trees. Young trees have a strong, vigorously growing central leader with a few upright growing branches. For fruit production it is an undesirable tree shape and mechanical devices are used to force the lateral branches to grow more horizontally. Promalin stimulates branching and increase the branch angle, as well as increase shoot elongation, all of which aid in the development of a scaffold branching system more suitable for fruit production (DAVIES, 1995).

2.3.4 Growth retardants

2.3.4.1 Nature and effects of growth retardants

Growth retardants are a diverse group of synthetic compounds that reduce stem elongation and generally increase the green colour of leaves. These compounds inhibit cell division in the subapical meristem of the shoot, but generally have little effect on the production of leaves or on root growth. The physiological effects of growth retardants can be reversed by application of gibberellins (DAVIES, 1995).

2.3.4.2 Commercial uses of growth retardants

2.3.4.2.1 Pinching agents

Some growth-retardant chemicals prevent growth of axillary branches. This includes maleic hydrazide and oxathiins. The chemicals dikegulac and chlorflurenol are potent inhibitors of tree growth, and also fall into this class of chemical pinching agents. The primary mode of action of these chemicals is to prevent cell development, disrupt differentiation of the meristem, and repress apical dominance (ARZEE *et al*., 1977).

2.3.4.2.2 Controlling of stem elongation in greenhouse crops

The application of growth retardants to potted plants results in shorter, more rigid stems and darker green foliage, characteristics that increase the value of the crop. In chrysanthemums, daminozide is effective as foliar spray and ancymidol may be used as both a foliar spray or a soil drench (LARSON and KIMMINS, 1971). In poinsettia, chlormequat chloride is used extensively for height control since it is less expensive than ancymidol. In Easter lily, ancymidol is used because it is the most effective compound for reducing stem height in this plant. Paclobutrazol (1-(4 chlorophenyl)4,4-dimethyl-2(1,2,4-triazol-1-yl)pentan-3-ol) and triazole fungicide triademefon will also control height, but higher concentrations are required in comparison to ancymidol (WULSTER *et al*., 1987).

2.3.4.2.3 Controlling vegetative growth

In a crop such as cotton and under certain conditions of high fertility and favourable environmental conditions, excessive vegetative growth results. Mepiquat chloride (1,1,dimethylpiperidinium chloride) applied at the time of flowering can reduce growth by 20-30%. Early yield of cotton is often increased by this treatment presumably due to greater light penetration into the canopy, thus allowing fruit set to take place in flowers produced on the lower nodes of the plant. Reduced vegetative growth also allows greater coverage of insecticides, fungicides and defoliants (HEILMAN, 1981).

2.3.4.2.4 Increasing fruit set

Application of chlormequat chloride to grapes before bloom increases fruit set of seeded berries (COOMBE, 1965). Cluster fresh weight is increased as a result of treatment. In addition to increasing cluster yield, vine growth is reduced by growth retardant treatment. It is not clear whether the increase in fruit set is due to a direct effect on this process by decreasing GA levels (GA is used for berry thinning) or an indirect effect resulting from decreased vegetative growth. Exceedingly vigorous shoot growth is often associated with poor fruit set in the field. Moreover, if shoot tips

are removed, fruit set in grape can be increased, and the growth retardants are not capable of further fruit set in detopped plants (DAVIES, 1995).

2.3.4.2.5 Induction of flower bud formation

Flowering can be stimulated in young apple trees by daminozide application and in pear trees by chlormequat chloride treatment. The growth retardants decrease shoot elongation in fruit trees, and perhaps by inhibiting vegetative growth, flower bud initiation is promoted (DAVIES, 1995).

2.3.4.2.6 Controlling tree size

Paclobutrazol and other triazole analogs are probably the most effective compounds for controlling shoot elongation in fruit trees (STEFFINS, 1988). Controlling tree size with these compounds will be an effective way of maintaining tree height for maximum spraying and harvesting efficiency in conjuction with modern pruning practices such as summer mowing of the tree canopy. Growth of woody landscape plants may also be effectively controlled by using the triazole, paclobutrazol and uniconazole (KEEVER *et al*., 1990).

2.4 SMOKE TECHNOLOGY

2.4.1 The role of smoke in horticulture

The effects of smoke and aqueous smoke extracts in the regulation of seed germination have been extensively examined (BROWN and VAN STADEN 1997; VAN STADEN *et al.,* 2000; LIGHT and VAN STADEN, 2004). Seeds can be pre-treated with smoke for use in horticulture, agriculture and in ecological rehabilitation. Smoke technology was used to promote the germination of many fynbos species for garden use and sale to the public. Lists of the fynbos species which have seeds that exhibit a good germination response to smoke were published by BROWN and BOTHA (2002). Prior to the use of smoke for promoting seed germination, many of

these species were difficult or impossible to propagate from seed. However, many of these species responded well to smoke treatments and are now available more readily for horticultural use (BROWN *et al*. 1994). The retention of the germination cue, once seeds have been exposed to smoke, allows for the pre-treatment and subsequent storage of seeds prior to sowing (BAXTER and VAN, STADEN 1994; BROWN and VAN STADEN, 1999). The germination enhancement of Australian species by smoke has been reported in more than 170 species from 37 families (ROCHE *et al.,* 1997a). A commercial product from smoke was developed and is marketed as 'Seed Starter', which is a concentrated smoke solution which is used in a diluted form (LIGHT and VAN STADEN, 2004).

2.4.1 The role of smoke in agriculture

Some vegetable crops, such as lettuce and celery (DREWES *et al.,* 1995; THOMAS and VAN STADEN 1995), have shown enhanced germination with smoke. Smoke treatments could possibly be used to promote synchronous germination of seeds, and to increase the rate of germination of certain agricultural crops. Seeds can be successfully pre-treated with smoke (LIGHT and VAN STADEN, 2004). Traditionally, rural subsistence farmers store their maize cobs over a fireplace in a hut. This indigenous method of maize storage thus causes the seeds to come into contact with large quantities of smoke. MODI (2002), using two traditional maize landraces, showed that the seeds exposed to smoke had a higher germination rate and final germination than untreated seeds. Furthermore, smoke-treated seeds produced significantly more vigorous seedlings than untreated seeds.

3 Response of *Jatropha curcas* plants to summer and winter manual pruning

3.1 INTRODUCTION

Judicious manipulation of growth and production cycles in fruit trees requires an understanding of the developmental and phenological features of the trees (NING *et al.,* 2004). Bud outgrowth is regulated by the interaction of environmental and endogenous signals, such as plant hormones. These interacting factors have a major influence on shoot system architecture. Understanding the origin of growth metamers and the relationship between preformation and final shoot morphology, is particularly important to understand the tree canopy (SPANN *et al.*, 2008).

Crop architecture plays an important role in *J. curcas,* whereby proper pruning helps in producing more branches and healthy inflorescences to enhance good fruit set and ultimately yield (GOUR, 2006). The pruning of terminals is essential at six months to induce lateral branch formation (GOUR, 2006; KUREEL, 2006). Pruning at 30 cm height is ideal to manage. Likewise the secondary and tertiary branches are to be pruned at the end of first year to induce a minimum of 25 branches and 35-40 branches at the end of the second year. During the second year each side branch should be pruned retaining 1/3rd of the branch on the plant. Periodic pruning can be carried out depending upon the vegetative growth of the plants (GOUR, 2006). However, the pruning should be done when the tree sheds leaves and enters into a period of dormancy, preferably during the winter season. The trees are kept short to be manageable during flowering and fruiting and to provide ease of movement during harvesting. Canopy management is advisable in trees with a terminal bearing. Plant types with a branch in every leaf axil should not be pruned vigorously. The entire plant has to be cut to ground level leaving only a 45 cm stump once in 10 years. The re-growth is quick and the pruned plants starts yielding in about a year. This procedure induces new growth and helps to stabilize yield (GOUR, 2006).

In this study, manual pruning was performed on two-year-old *J. curcas* plants under summer and winter conditions. The aim was to determine which pruning time would most likely improve branching, shape and yield.

3.2 MATERIALS AND METHODS

3.2.1 Study site

The experiments were conducted on *J. curcas* L. plants growing at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga), Pietermaritzburg, South Africa (30˚41' E, 29˚67' S; and 781 m asl).

3.2.2 The experiments

The experiments were performed on two-year-old plants of *J. curcas* established from seeds. Plants were of the same variety and similar in height. For both summer- and winter-pruning experiments, manual pruning (MP) was performed using a lopping shear by cutting the plant's leading middle stalk near ground level and the remaining of the branches 45- 50 cm above ground level. The cut was made about 1 cm above an active bud to prevent dieback of the stem and to encourage a new branch to develop (**Figure 3.1 D**). The manual pruning was assigned to 16 plants in each of three plots which gives a total of 48 plants (n = 48) for each of the two experiments (**Table 3.1**). On each plot, 16 plants were left un-pruned, representing the control, giving a total of 48 plants (n = 48) for each experiment (**Table 3.1**). Summer pruning was performed on six consecutive days between 22 – 28 March 2007. Winter pruning was performed on four consecutive days between 20 – 24 August 2007.

Table 3.1 Summary of the design of the manual pruning experiment.

Plot	Distance from plot I (M)	No. of plants			
		Summer pruning		Winter pruning	
		control	pruned	control	pruned
I	0	16	16	16	16
II	300	16	16	16	16
II	600	16	16	16	16
Total		48	48	48	48
Grand total		96		96	

Three parameters were used to compare the differences between the two types of pruning:

(i) Branching which was determined as the increase in the branch number over the initial number of branches before pruning. Data were collected between 10-16 January 2008;

(ii) Plant crown diameter, which was determined as the increase over the initially measured crown diameter of the plants before pruning. The crown diameter was determined by measuring the width of the plant shoot using a metal ruler; and

(ii) Number of fruit per plant which was determined by counting the number of fruits harvested from each plant. Fruit harvesting for both experiments was done between 19 February – 23 April 2008.

3.2.3 Statistical analysis

Branching and crown diameter was considered as the increase in the number of branch and crown width over the initial readings. SPSS® release 15 statistical software was used for data analysis using one-way ANOVA. Tukey's test was used to determine the differences between treatments (SPSS Inc., Chicago, USA).

3.3 RESULTS

Summer- and winter-pruning produced plants with more branches compared to the un-pruned control (**Figure 3.2A)**. However, no significant differences were found in branching between summer- and winter-pruning (**Figure 3.2A).** Winter-pruning produced plants with wider crowns compared to the summer-pruning and to the un-pruned control plants (**Figure 3.2B)**. The control plants, however, produced more fruit per plants compared to the summer- and winter-pruned plants (**Figure 3.2C)**. No significant differences in number of fruit per plants were detected between winter- and summer-pruning. However, summer-pruned plants mainained a production level slightly higher than winter-pruned plants (**Figure 3.2C)**.

Figure 3.1 Response of *Jatropha curcas* plants to summer- and winter-manual pruning. Control of un-pruned plants shade leaves in winter (A and B); Sprouting of new branches after pruning (C and D); Plant shape in summer-pruned plants (E); Plant shape in winter-pruned plants (F).

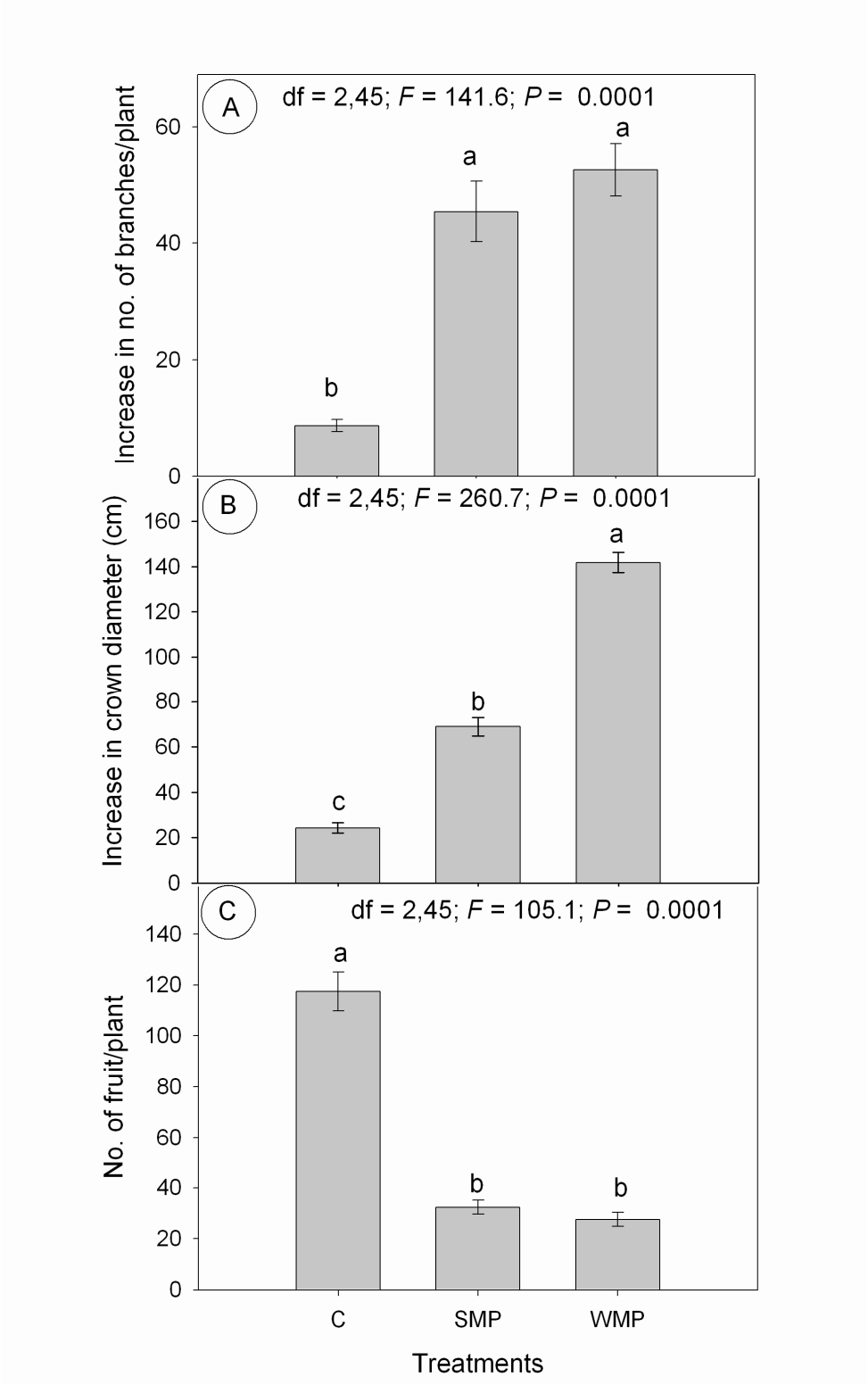

Figure 3.2 Branching, crown diameter and number of fruit per plant in two-year-old plants of *Jatropha curcas* in the subsequent season following summer- and winter-manual pruning, C ≡ control, SMP ≡ Summer-manual pruning, WMP ≡ winter-manual pruning. S.E bars with the same letters are not significantly different to each other according to Tukey's test at $P < 0.05$.

3.4 DISCUSSION

Shoot branching is the process by which auxiliary buds, located on the axil of a leaf develop and form new flowers or branches. The process by which a dormant bud activates and becomes an actively growing branch is complex and very finely tuned. Bud outgrowth is regulated by the interaction of environmental signals and endogenous ones, such as plant hormones. These interacting factors have a major influence on shoot system architecture. Shoot growth of woody plants may be either (1) preformed where, the metamers composing a shoot are differentiated in dormant bud, or (2) neoformed where, the metamers are not entirely differentiated in the dormant bud and a portion of vegetative growth is differentiated during the growing season. Understanding the origin of growth metamers and the relationship between preformation and final shoot morphology, is particulary important to understand the tree canopy (SPANN *et al.*, 2008).

It has been reported that manual pruning of *J. curcas* plants is preferable and efficient during the dormant stage of growth (GOUR, 2006). This study, however, was motivated by: (1) the needs to compare the performance of *J. curcas* plants during the late summer and late winter under the study site weather conditions; (2) the need for obtaining comparable information about the manual pruning performance in a bigger sample size simultaneously with the other experiments testing for the effect of chemical pruning (**Chapter 4**).

The results show that summer- and winter-pruning produced plants with more branches compared to the un-pruned control plants (**Figure 3.2A)**. However, no differences were found in branching between summer- and winter-pruning (**Figure 3.2A**). Stimulation of shoot growth by pruning is well documented for most of the orchard and fruit trees. It was reported that for most deciduous trees, pruning at the distal end of shoots releases more proximal lateral vegetative buds from apical control, allowing more shoots to grow. However, according to pruning dogma, during the first year following pruning, individual shoots are invigorated, but intra-shoot competition due to the stimulation of lateral bud growth may limit the total growth (HARRIS *et al.,* 1983; SPAAN *et al.,* 2008). Therefore, SPANN (2008) hypothesised

that in *Pistacia vera* total shoot growth would be greatest in the first year following pruning, that individual shoots would be vigorous and competition would be negligible. That would then be followed, in the next year, by a decrease in total growth as neoformation decreased and a higher percentage of the total growth was preformed.

The study showed that winter-pruning had a wider crown diameter compared to summer-pruning and un-pruned control plants (**Figure 3.2B**). Increased vegetative growth, however, is not necessarily a benefit in mature tree crops, as it may not be correlated specifically with increased yield (JOHNSON and HANDLEY, 2000; SPANN *et al.* 2008). Indeed, the results show that no variations in number of fruit per plants were detected between summer- and winter-pruning and that yield of pruned plants was significantly lower than that of control plants (**Figure 3.2C)**.

The control plants produced more fruit per plant compared to both summer- and winter-pruned plants (**Figure 3.2C)**. The explanation of these results is that the reduction of yield in both pruning treatments may be due to the change in the tree size and structure after pruning. Further, the movement of carbohydrates and their accumulation in different parts of plants are affected by environmental conditions and plant treatments. Thus shoot light penetration and distribution within the canopy are affected by pruning (CALATAYUD *et al.,* 2008). Furthermore, LI *et al.* (2003) stated that in pruned plants canopy size might result in less light interception and decreasing canopy photosynthetic efficiency and consequently decreased yield. These results are also in line with KÜDEN and SON (2000) who found that in apricot the unpruned contol plants were higher in carbohydrate content compared to the pruned plants in the first year.

4 Promoting branching of *Jatropha curcas* by foliar application of plant growth regulators

4.1 INTRODUCTION

Declining availability of fossil fuels is driving the current search for alternative sources of energy. Biofuels offer promise, but are controversial because of the large land area required for production, potential for competition with food production, and their marginal economic viability in the absence of subsidies (GRESSEL, 2008). These potential negative impacts could be reduced and profitability increased if production could be made more efficient. A crop with potential for biofuel production in arid and semi-arid regions is the physic nut, *Jatropha curcas* L. (HELLER, 1996; SHARMA, 2006). It was suggested that the oil yield from *J. curcas* nuts can be improved if the number of seed bearing branches could be increased. The pruning of apical buds of the main stem of one-year-old plants can increase the number of main and secondary branches (KUREEL, 2006). Proper pruning of *J. curcas* helps in producing more branches with healthy inflorescences. This enhances flowering and fruit set that ultimately increases yield (GOUR, 2006). However, the cost, convenience and efficiency of manual pruning in large-scale plantations still remains a major concern.

A number of plant growth regulators (PGRs) can serve as powerful tools for manipulating tree growth and yield (LOVAT, 2006). The cytokinin BA (6-benzyladenine) is known to release apical dominance and promote new lateral branches (SVENSON, 1991) by altering the auxin to cytokinin ratio in shoot tips (CLINE, 1988). The auxin transport inhibitor TIBA (2,3,5-triiodobenzoic acid) promotes the activation of auxiliary buds under a wide range of experimental and field conditions (MOREY *et al*., 1975). Dikegulac (DK) (2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid) is used to overcome apical dominance and increase axillary shoot production (BANKO and STEFANI, 1996). Maleic hydrazide (MH) (1,2-dihydro-3,6-pyridazinedione, coline salt) acts as an anti-auxin or a regulator of auxin metabolism (HOFFMAN and PARUPS, 1964), suggesting the possibility that it may increase cytokinin levels in lateral buds and stimulate shoot elongation (ITO *et al*.,

2000). This study was undertaken to evaluate the effect of these proven and widely used PGRs on branching of *J. curcas* plants as a possible substitution to manual pruning.

4.2 MATERIALS AND METHODS

4.2.1 Shade house experiment

This experiment was conducted on five-month-old *J. curcas* plants in a shade house of the University of KwaZulu-Natal Botanical Garden, Pietermaritzburg, South Africa. The mean solar radiation at mid-day in the shade house was 331 µmol $m^{-2}s^{-1}$. The soil mixture in each plastic pot (20 cm) was compost: bark (chipped and decomposed pine): LAN (limestone ammonium nitrate): 2:3:2 NPK (nitrogen, phosphorus, potassium) (4:1:0.1:0.1). The plants used in this experiment were all of the same variety with similar height and stem diameter. The foliar treatments consisted of BA (3, 6, 9, 12 and 15 mmol l^{-1}), TIBA (0.5, 1.0, 1.5 and 2 mmol l^{-1}), DK (2, 4, 6 and 8 mmol l^{-1}) and MH (2, 3 and 4 mmol l^{-1}). A small volume of sodium hydroxide (0.1 M) was used to solubilize the PGRs before adding water. Plants sprayed with distilled water + an equivalent amount of 0.1 M NaOH served as control. A few drops of Tween® 20 (Merck) were added as surfactant. Plant growth regulators BA, TIBA and DK were purchased from Sigma-Aldrich Ltd., South Africa, and MH was obtained from Koch-Light Laboratories Ltd., U.K. The plants were treated once on 20 October 2007 (foliar application of 50 ml of test solution per seedling) using a new plastic sprayer (500 ml) for each plant growth regulator. Manual pruning was done on the same day as the foliar treatments. Each treatment consisted of sixteen plants considering a single plant as one replicate, arranged randomly. The distance between any two pots was 45 cm. Plant height, shoot length, number of lateral branches and leaves were recorded before and after treating (one and four months) the plants. Growth of a lateral branch was considered when it had elongated more than 3 cm.

4.2.2 Field experiment

The treatments were the same as described above for one-year-old field-grown *J. curcas* plants. The experiment was conducted at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga), Pietermaritzburg, South Africa (Latitude 30˚41' E, Longitude 29˚67' S and Altitude 781 m asl). Each plant received 200 ml of respective test solution on 20 May 2007. Manual pruning was done on the same day as that of the foliar treatment. Each treatment consisted of twelve plants, considering a single plant as one replicate, selected randomly. The distance between the plants was 2.5 m. Plant height, plant crown diameter, number of lateral branches and stem diameter at the base were recorded before and after treatments (three and seven months).

4.2.3 Data analysis

Data were analyzed using SPSS® version 15 (SPSS Inc., Chicago, USA) statistical software. Effect of treatments on plant growth was analyzed using one-way analysis of variance (ANOVA). Tukey's test was used in order to compare the significance of differences among treatments.

4.3 RESULTS

4.3.1 Shade house experiment

Foliar applications of BA significantly increased the number of branches after one and four months in comparison to the control where no new branches developed (**Figure 4.1C and Figure 4.3A**). The number of leaves produced by plants treated with BA at 15 mmol l^{-1} was significantly higher than the control after one month (**Table 4.1**). Of all the concentrations of TIBA tested, only 1.5 mmol l^{-1} led to a significant increase in the number of branches (1.6) compared to the control, with no branching after four months. On the other hand, manual pruning produced significantly more branches (1.8) than the control and other concentrations of TIBA applied (**Figure 4.1D and Figure 4.3B**). After one and four months, TIBA application

did not significantly improve plant height, shoot length and number of leaves compared to the control plants (**Table 4.2**). Spraying of plants with DK (6 mmol l^{-1}) and MH (2 mmol l^{-1}) resulted in a significantly greater number of branches (4.7 and 2.8 respectively) compared to control plants after four months (**Figure 4.1E and Figure 4.3C**). A high concentration of DK (8 mmol l^{-1}) and MH (4 mmol l^{-1}) significantly decreased plant height, shoot length and number of leaves in comparison to the untreated plants after four months (**Table 4.3** and **Table 4.4** respectively). There were some growth abnormalities such as short yellowish leaves and stunted plants in DK treatments of higher concentration.

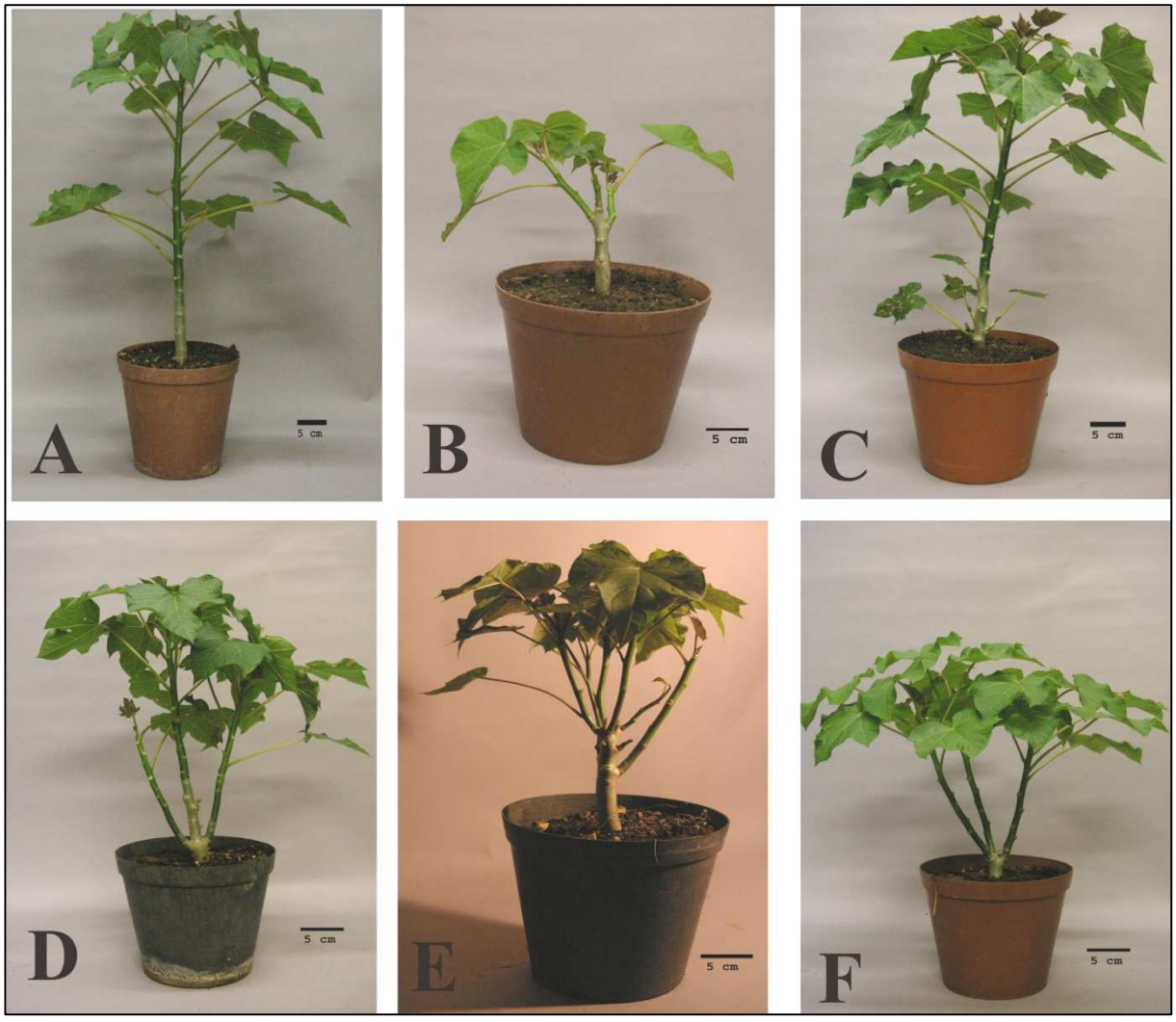

Figure 4.1 Influence of foliar application of plant growth regulators on lateral branching of *Jatropha curcas* plants (five-month-old) under shade house conditions four months after treatment. (A) Control; (B) Manual Pruning; (C) BA 12 mmol l^{-1}; (D) TIBA 1.5 mmol l^{-1}; (E) DK 6 mmol l^{-1}; (F) MH 2 mmol l^{-1}. Bar scale = 5 cm.

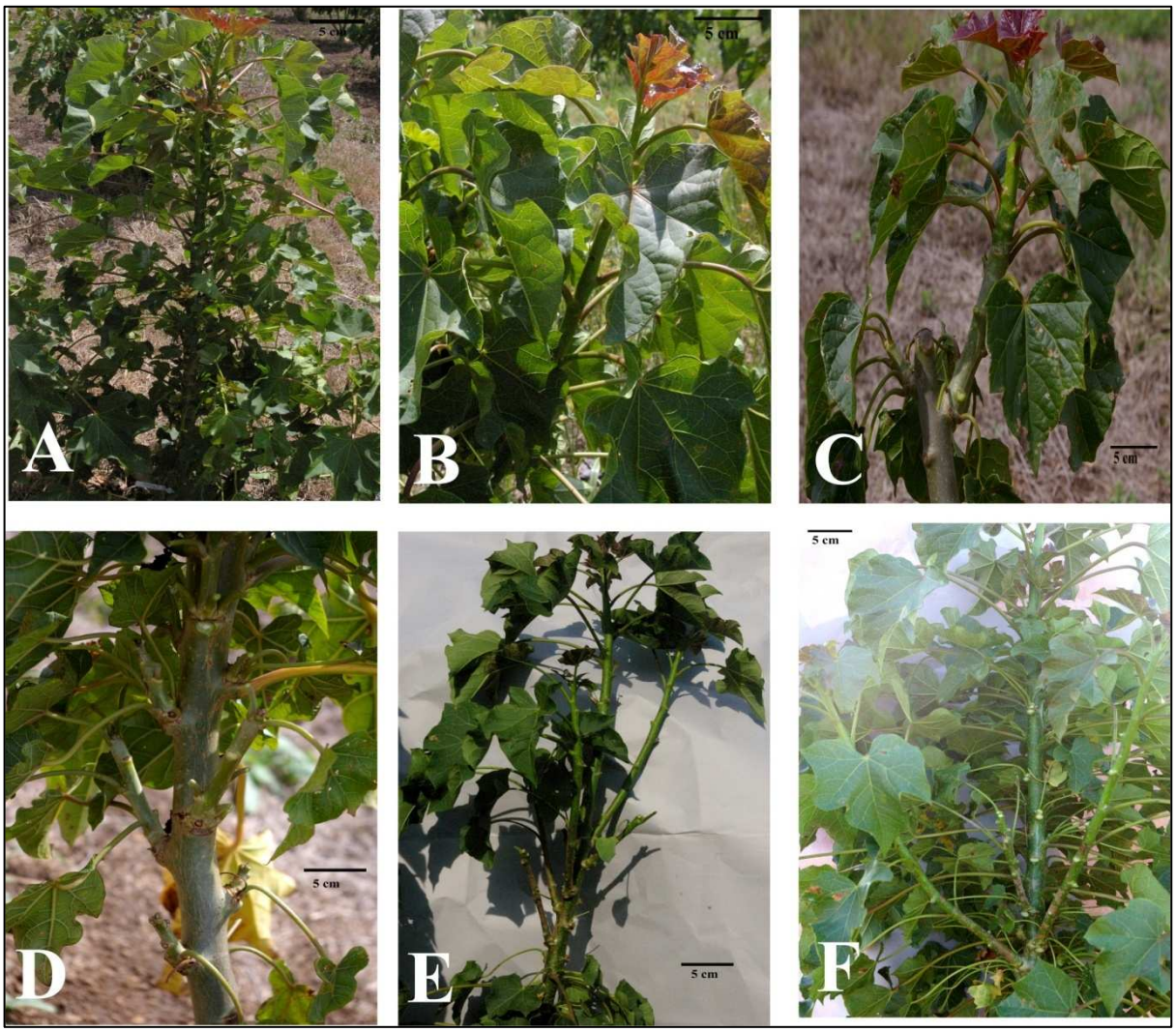

Figure 4.2 Influence of foliar application of plant growth regulators on lateral branching of *Jatropha curcas* plants (one-year-old) under field conditions seven months after treatment. (A) Control; (B) TIBA 1.5 mmol l^{-1}; (C) Manual Pruning; (D) BA 12 mmol l^{-1}; (E) DK 6 mmol l^{-1}; (F) MH 2 mmol l^{-1}. Bar scale = 5 cm.

4.3.2 Field experiment

One-year-old plants treated with BA at 12 mmol l^{-1} produced a significantly higher number of branches after three and seven months (5.5 and 13 .2 respectively) than the untreated plants (1.2 and 3.8 respectively) (**Figure 4.2C and Figure 4.4A**). Manual pruning showed no significant increase in the number of branches compared to this BA concentration after seven months (**Figure 4.4A**). Although some concentrations of BA tested increased plant height, crown- and stem diameter, these results were not significantly different from those obtained for the control (**Table 4.1**). A foliar application of TIBA at 1 and 2 mmol l^{-1} produced a significantly greater number of branches (15.9 and 15 respectively) compared to the control (3.8) and manually pruned (5.7) plants after seven months (**Figure 4.2D and Figure 4.4B**). Plant height, crown- and stem diameter were not significantly higher than the control with the application of TIBA (**Table 4.2**). A concentration of 2 mmol l^{-1} of DK produced more branches (18.1) compared to the control (3.8) and manual pruning (5.7) seven months after foliar application (**Figure 4.2E and Figure 4.4C**). Maleic hydrazide at 3 mmol l^{-1} produced a significantly greater number of branches (11.7) compared to the control (3.8) and manual pruning (5.7) after seven months of foliar application (**Figure 4.2F and Figure 4.4.4D**). However, plant height, crown- and stem diameter did not significantly increase when compared to the control plants (**Table 4.4**).

Table 4.1 Effects of a single foliar application of benzyladenine (BA) and manual pruning (MP) on different growth parameters of *Jatropha curcas*. Mean values ± S.E. with no letters are not significantly different. Mean values ± S.E. with different letter(s) are significantly different at P < 0.05 (Tukey's test).

Treatment	Five-month-old plants (shade house conditions)			Twelve-month-old plants (field conditions)		
BA (mmol l^{-1})	Plant height (cm)	Shoot length (cm)	Leaves (no.)	Plant height (cm)	Crown diameter (cm)	Stem diameter (cm)
	One month after spraying			Three months after spraying		
0	19.2 ± 8.8^{ab}	2.9 ± 0.8	9.7 ± 2.4^{b}	5.5 ± 1.6	18.4 ± 6.9^{ab}	3.3 ± 1.0
3	25.0 ± 5.7^{a}	3.3 ± 0.6	15.5 ± 2.1^{b}	7.0 ± 1.6	14.3 ± 5.3^{ab}	1.9 ± 0.5
6	9.5 ± 2.2^{ab}	1.7 ± 0.2	12.9 ± 2.3^{b}	3.3 ± 0.6	17.9 ± 8.1^{ab}	2.8 ± 0.8
9	3.1 ± 1.1^{b}	0.7 ± 0.5	10.6 ± 2.3^{b}	6.0 ± 1.8	28.7 ± 11^{a}	3.5 ± 0.7
12	3.4 ± 0.5^{b}	1.24 ± 0.8	13.8 ± 2.4^{b}	9.2 ± 2.4	16.6 ± 4.6^{ab}	3.0 ± 0.6
15	3.5 ± 2.9^{b}	4.2 ± 0.3	26.2 ± 3.2^{a}	----	---	---
MP	10.0 ± 3.7^{ab}	1.6 ± 0.5	9.9 ± 1.5^{b}	4.0 ± 1.2 a	5.1 ± 0.6^{b}	2.1 ± 0.6
	Four months after spraying			Seven months after spraying		
0	32.8 ± 2.2^{a}	24.4 ± 1.8^{a}	32.8 ± 2.2^{a}	54.5 ± 9.8^{a}	220 ± 22	2.6 ± 0.3^{ab}
3	34.5 ± 2.5^{a}	27.5 ± 2.1^{a}	18.8 ±2.5^{b}	52.6 ± 7.8^{a}	231 ± 22	1.9 ± 0.2^{ab}
6	16.7 ± 5.2^{b}	11.0 ± 3.7^{bc}	13.8 ± 1.6^{bc}	61.2 ± 6.1^{a}	241 ± 18	2.3 ± 0.1^{ab}
9	10.2 ± 2.4^{b}	9.6 ± 1.9^{bc}	17.5 ± 1.5^{b}	63.4 ± 4.2^{a}	244 ± 22	2.9 ± 0.3^{a}
12	16.6 ±1.6^{b}	12.6 ± 1.9^{bc}	13.8 ± 3.2^{b}	75.4 ± 5.7^{a}	235 ± 19	2.9 ± 0.1^{a}
15	14.9 ± 3.4^{b}	20.1 ± 2.2^{ab}	17.4 ± 3.8^{b}	---	---	---
MP	3.7 ± 3.9^{b}	2.3 ± 1.8^{c}	3.7 ± 3.3^{c}	16.6 ± 13.6^{b}	142 ± 40	1.6 ± 0.3^{b}

Table 4.2 Effects of a single foliar application of 2,3,5-triiodobenzoic acid (TIBA) and manual pruning (MP) on different growth parameters of *Jatropha curcas*. Mean values ± S.E. with no letters are not significantly different. Mean values ± S.E. with different letter(s) are significantly different at P < 0.05 (Tukey's test).

Treatment TIBA (mmol l^{-1})	Five-month-old plants (shade house conditions)			Twelve-month-old plants (field conditions)		
	Plant height (cm)	Shoot length (cm)	Leaves (no.)	Plant height (cm)	Crown diameter (cm)	Stem diameter (cm)
	One month after spraying			Three months after spraying		
0	19.2 ± 8.8	2.9 ± 0.8	9.7 ± 2.4	5.5 ± 1.6	18.4 ± 6.9^{a}	3.3 ± 1.0
0.5	21.5 ± 6.9	2.1 ± 0.5	7.4 ± 2.0	6.3 ± 1.5	17.3 ± 6.0^{a}	3.5 ± 0.9
1.0	32.7 ± 10.7	2.5 ± 0.9	5.9 ± 2.5	9.0 ± 2.4	26.2 ± 8.5^{a}	3.7 ± 0.6
1.5	16.2 ± 5.0	3.6 ± 1.0	7.5 ± 2.2	5.0 ± 1.1	39.9 ± 13.4^{a}	3.4 ± 0.7
2.0	15.8 ± 4.8	3.5 ± 1.0	7.2 ± 1.3	6.5 ± 0.9	24.5 ± 9.3^{a}	2.2 ± 0.6
MP	10.0 ± 3.7	1.6 ± 0.5	9.9 ± 1.5	4.0 ± 1.2	5.1 ± 0.6^{b}	2.1 ± 0.6
	Four months after spraying			Seven months after spraying		
0	32.8 ± 2.2^{a}	24.4 ± 1.8^{a}	32.8 ± 2.2^{a}	54.5 ± 9.8	220 ± 22	2.6 ± 0.3
0.5	27.2 ± 2.5^{ab}	22.4 ± 2.1^{ab}	22.4 ± 2.5^{a}	39.4 ± 8.6	228 ± 35	1.6 ± 0.3
1.0	15.8 ± 3.5^{b}	10.3 ± 2.9^{b}	8.7 ± 3.7^{b}	52.0 ± 3.8	236 ± 23	1.7 ± 0.5
1.5	16.4 ± 4.2^{b}	15.6 ± 3.8^{ab}	20.6 ± 2.8^{a}	53.0 ± 5.0	258 ± 18	2.7 ± 0.2
2.0	21.1 ± 2.7^{ab}	22.2 ± 2.9^{ab}	15.4 ± 3.7^{b}	27.0 ± 14.6	251 ± 25	2.4 ± 0.2
MP	3.7 ± 2.5^{c}	2.3 ± 2.1^{c}	3.7 ± 3.3^{c}	16.6 ± 13.6	142 ± 40	1.6 ± 0.3

Table 4.3 Effects of a single foliar application of 2,3:4,6-di-oisopropylidene-2-keto-L-gulonic acid sodium salt (Dikegulac) (DK) and manual pruning (MP) on different growth parameters of *Jatropha curcas*. Mean values ± S.E. with no letters are not significantly different. Mean values ± S.E. with different letter(s) are significantly different at P < 0.05 (Tukey's test).

Treatment DK (mmol l^{-1})	Five-month-old plants (shade house conditions)			Twelve-month-old plants (field conditions)		
	Plant height (cm)	Shoot length (cm)	Leaves (no.)	Plant height (cm)	Crown diameter (cm)	Stem diameter (cm)
	One month after spraying			Three months after spraying		
0	19.2 ± 8.8^{ab}	2.9 ± 0.8^{a}	9.7 ± 2.4^{a}	5.5 ± 1.6	18.4 ± 6.9^{ab}	3.3 ± 1.0
2	31.3 ± 7.5^{a}	0.83 ± 0.4^{b}	6.3 ± 1.6^{ab}	6.2 ± 1.4	18.0 ± 4.38^{ab}	2.1 ± 0.5
4	10.1 ± 4.1^{b}	1.9 ± 0.8^{a}	10.7 ± 3.5^{a}	5.3 ± 1.4	19.9 ± 8.4^{a}	3.3.8 ± 0.8
6	10.9 ± 3.2^{ab}	1.2 ± 0.5^{ab}	8.4 ± 4.2^{a}	2.58 ± 0.6	7.53 ± 1.66^{ab}	2.7 ± 0.7
8	3.4 ± 1.5^{c}	0.66 ± 0.2^{b}	4.8 ± 2.4^{b}	2.75 ± 0.7	6.96 ± 1.13^{ab}	2.8 ± 0.6
MP	10.0 ± 3.7^{b}	1.6 ± 0.5^{ab}	9.9 ± 1.5^{a}	4.0 ± 1.2	5.1 ± 0.6^{b}	2.1 ± 0.6
	Four months after spraying			Seven months after spraying		
0	32.8 ± 2.2^{ab}	24.4 ± 1.8^{a}	32.8 ± 2.2^{a}	54.5 ± 9.8^{ab}	220 ± 22^{ab}	2.6 ± 0.3^{ab}
2	29.5 ± 2.5^{ab}	22.8 ± 2.1^{ab}	10.4 ± 2.5^{c}	74.7 ± 33.9^{a}	283 ± 37^{a}	4.0 ± 0.4^{a}
4	14.6 ± 2.6^{b}	11.3 ± 3.7^{b}	21.8 ± 1.5^{b}	26.7 ± 47.3^{b}	223 ± 17^{ab}	2.4 ± 0.4^{b}
6	18.5 ± 2.4^{ab}	17.0 ± 1.9^{ab}	18.0 ± 2.8^{bc}	47.7 ± 32.3^{ab}	245 ± 28^{ab}	2.1 ± 0.3^{b}
8	2.2 ± 4.3^{c}	1.2 ± 2.3^{c}	6.0 ± 5.5^{cd}	54.4 ± 44.1^{ab}	239 ± 26^{ab}	2.5 ± 0.3^{ab}
MP	3.7 ± 2.0^{c}	2.3 ± 2.1^{c}	3.7 ± 3.3^{d}	16.6 ± 13.6^{b}	142 ± 40^{b}	1.6 ± 0.3^{b}

Table 4.4 Effects of a single foliar application of maleic hydrazide (MH) and manual pruning (MP) on different growth parameters of *Jatropha curcas*. Mean values ± S.E. with no letters are not significantly different. Mean values ± S.E. with different letter(s) are significantly different at P < 0.05 (Tukey's test).

Treatment MH (mmol l^{-1})	Five-month-old plants (shade house conditions)			Twelve-month-old plants (field conditions)		
	Plant height (cm)	Shoot length (cm)	Leaves (no.)	Plant height (cm)	Crown diameter (cm)	Stem diameter (cm)
	One month after spraying			Three months after spraying		
0	19.2 ± 8.8^{a}	2.9 ± 0.8^{a}	9.7 ± 2.4^{a}	5.5 ± 1.6	18.4 ± 6.9^{ab}	3.3 ± 1.0
2	5.24 ± 1.6^{b}	1.2 ± 0.3^{ab}	3.1 ± 0.96^{b}	5.8 ± 2.3	19.77 ± 5.3^{a}	2.9 ± 0.9
3	8.41 ± 3.2^{ab}	0.25 ± 0.1^{b}	0.3 ± 0.21^{c}	5.1 ±1.4	5.33 ± 8.1^{ab}	2.8 ± 0.6
4	16.68 ± 1.1^{a}	0.55 ± 0.3^{b}	0.3 ± 0.5^{c}	2.8 ± 0.6	4.75 ± 0.7^{b}	3.1 ± 0.4
MP	10.0 ± 3.7^{ab}	1.6 ± 0.5^{ab}	9.9 ± 1.5^{a}	4.0 ± 1.2	5.1 ± 0.6^{b}	2.1 ± 0.6
	Four months after spraying			Seven months after spraying		
0	32.8 ± 2.2^{a}	24.4 ± 1.8^{a}	32.8 ± 2.2^{a}	54.5 ± 9.8^{ab}	220 ± 22	2.6 ± 0.3
2	25.4 ± 2.5^{a}	17.7 ± 2.1^{ab}	20.6 ± 2.5^{b}	57.5 ± 5.5^{a}	239 ± 33	2.1 ± 0.3
3	13.7 ± 3.1^{b}	11.9 ± 1.9^{bc}	18.2 ± 2.1^{b}	43.0 ± 6.6^{ab}	215 ± 24	2.4 ± 0.4
4	10.9 ± 2.3^{b}	13.2 ± 3.5^{bc}	20.5 ± 2.7^{b}	45.4 ± 4.9^{ab}	217 ± 17	2.3 ± 0.2
MP	3.7 ± 2.0^{b}	2.3 ± 2.1^{c}	3.7 ± 3.3^{c}	16.6 ± 13.6^{b}	142 ± 40	1.6 ± 0.3

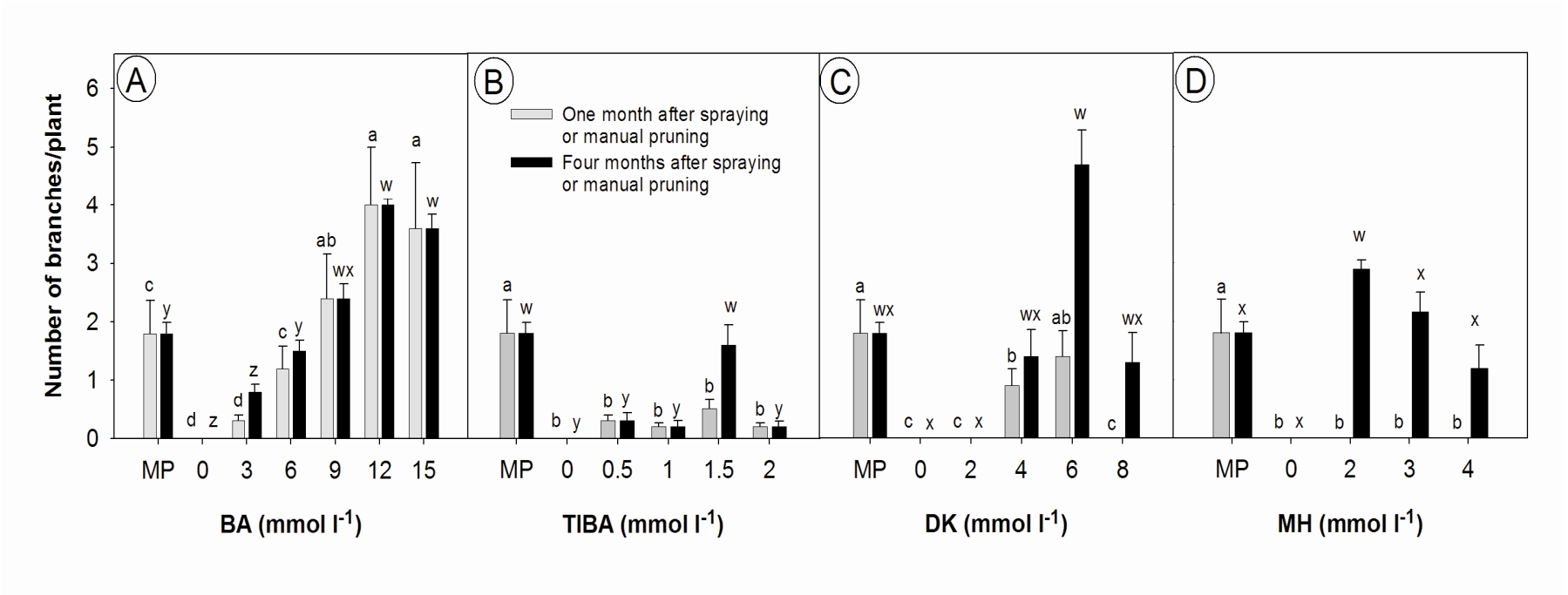

Figure 4.3 Influence of foliar application of plant growth regulators on lateral branching of *Jatropha curcas* plants (five-month-old) under shade house conditions. MP ≡ manual pruning and 0 ≡ control. Standard error (±) bars with different letter(s) are significantly different to each other according to Tukey's test ($P < 0.05$).

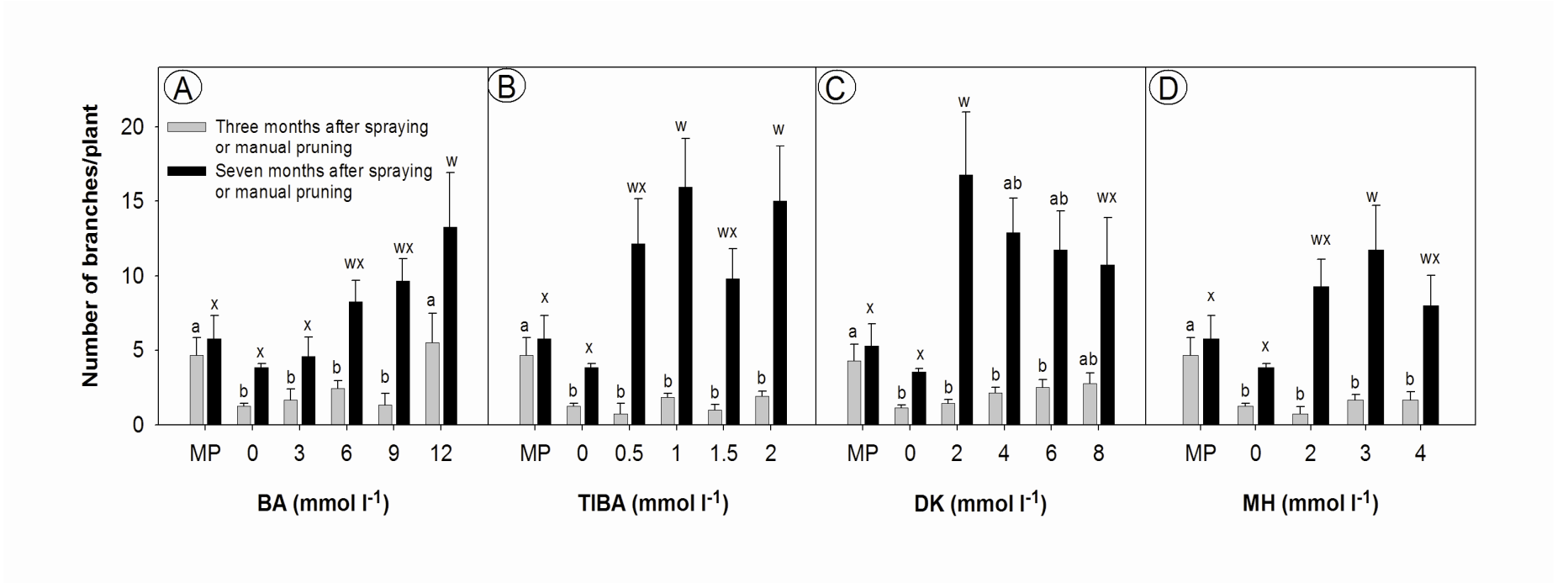

Figure 4.4 Influence of foliar application of plant growth regulators on lateral branching of *Jatropha curcas* plants (twelve-month-old) under field conditions. MP ≡ manual pruning and 0 ≡ control. Standard error (±) bars with different letter(s) are significantly different to each other according to Tukey's test ($P < 0.05$).

4.4 DISCUSSION

Apical dominance can be interrupted in several ways. One way is to reduce the internal ratio of auxin to cytokinin by applying external cytokinins. A second way is to apply a chemical that inhibits auxin production or transport. A third way is to kill the apical meristem which halts auxin production (BANGERTH *et al.,* 2000).

In the shade house, foliar application of BA (12 mmol l^{-1}) to five-month-old plants of *J. curcas* was very effective in producing a maximum number of lateral branches (**Figures 4.1C and 4.3A**). Similarly, SANSBERRO *et al*. (2006) showed an increase in number of branches of *Ilex paraguariensis* St. Hil. seedlings treated with foliar applications of BA. At most of the concentrations tested, BA decreased the plant height and shoot length of *J. curcas* grown under shade house conditions. These results are similar to the findings of HENNY (1986) who reported that BA increased lateral branches and decreased plant height of *Peperomia obtusifolia* L., resulting in shorter and more compact plants. Exogenous applications of BA had positive effects on the shoot growth of *Welkeri dieffenbachia* (WILSON and NELL, 1983) and *Pinus* species (BOE, 1990). Under field conditions, after seven months following BA treatment (12 mmol l^{-1}) there was a significant improvement in number of branches in *J. curcas* (**Figures 4.2D and 4.4A**). However, unlike the results of the shade house experiment, there was a non-significant increase in plant height and crown diameter with most concentrations of BA applied. This result suggests that the growth of *J. curcas* plants when treated with BA may differ under shade house and field conditions.

Foliar application of TIBA to *J. curcas* plants at 1.5 mmol l^{-1} under shade house conditions (after four months) and 1 mmol l^{-1} under field conditions (after seven months) yielded more branches than the control (**Figure 4.3B** and **Figure 4.4B** respectively). With both these growth conditions this effect was not immediate as the number of branches did not improve at early growth stages. Similarly, primary shoot development was delayed in *I. paraguariensis* with TIBA treatment (SANSBERRO *et al*., 2006). In the shade house, after four months from treatment, some concentrations of TIBA inhibited height, shoot length and number of leaves of *J. curcas* plants. However, under field conditions there was no significant effect.

In comparison to the shade house-treated plants, *J. curcas* plants that were treated in the field with DK (2 mmol l^{-1}) yielded a maximum number of branches after seven months (**Figure 4.3C** and **Figure, 4.4B**). This indicates that under field conditions *J. curcas* plants responded much better to a lower concentration of DK (2 mmol l^{-1}) than at an higher concentration (6 mmol l^{-1}) in the shade house for enhancing the number of lateral branches. This positive effect of a low concentration of DK in the field may be associated with the age and maturity of *J. curcas* plants. Increasing concentrations of DK decreased plant height, shoot length and number of leaves when compared to untreated plants in the shade house after four months. Similar results were reported for *Lonicerax heckrotti* Rehd. (BRUNER *et al*., 2000) and *Hedra helix* (AL-JUBOORY and WILLIAMS, 1991) where DK increased the number of shoots and decreased shoot length. This effect was not consistent in field-treated plants. The observation of abnormal growth of *J. curcas* plants in pots (shade house) caused by high concentration of DK supports the findings of SANSBERRO *et al*. (2006) that phytotoxicity was concentration dependent in *Ilex paraguariensis*. Similar observations were also reported by BANKO and STEFANI (1996) who noticed slight chlorosis and leaf deformity in *Salvia farinacea* with foliar application of DK. In this study, foliar application of a low concentration of DK (2 mmol l^{-1}) did not show any growth abnormalities in the field suggesting, that low concentrations of DK can be safely used in the field to promote branching of *J. curcas* plants.

Maleic hydrazide increased the number of lateral branches when compared to manual pruning and control plants after four and seven months under shade house (2 mmol l^{-1}) and field conditions (3 mmol l^{-1}) respectively (**Figure 4.3D** and **Figure, 4.4D**). These results indicate that the positive effect of MH on branching is only observed after a longer period, as in both cases there were no improvement at early growth stages. In the shade house after four months, MH (4 mmol l^{-1}) suppressed plant height and number of leaves with some growth abnormalities. Studies have revealed that MH-treated plants lose or show impaired apical dominance (NAYLOR and DAVIS, 1950). On the other hand, no significant effect was noticed in the field grown plants which exhibited normal growth.

5 Pollinator effectiveness, breeding system, and tests for inbreeding depression in *Jatropha curcas*

5.1 INTRODUCTION

Jatropha curcas L. is a monoecious crop with potential for biofuel production in arid and semi-arid regions (HELLER, 1996). *Jatrpha curcas* oil contains about 14% free fatty acid (FFA), which is well beyond the limit of 1% FFA level that can be converted into biodiesel by trans-esterification using an alkaline catalyst (TIWARI *et al*., 2007). The fatty acid methyl ester of its oil was found suitable for use as biodiesel as it meets the specifications of the International Biodiesel Standards (AZAM *et al*., 2005). However, its use is controversial because of the large land area required as yields (0.6 – 3 t/ha) are not yet economically viable.

Augmentation of agricultural pollination is a possible solution to increase profit by increasing seed yield. In many crops, the number of bee visits to receptive flowers can be a limiting step in obtaining optimal yields (ROLDÁN-SERRANO and GUERRA-SANZ, 2005). This is particularly true for crops that are not capable of autonomous selfing, or apomixis. Crops with unisexual flowers would be expected to be highly reliant on specific pollinators (WESTERKAMP and GOTTSBERGER, 2000). In commercial plantations of avocado (*Persea americana* Mill.), honeybees have been used successfully, and almost exclusively, for pollination. There was a strong positive correlation between honeybee activity in an avocado orchard, fruit set, and yield (VITHANAGE, 1990; ISH-AM *et al*., 1999). In *Coffea canephora,* fruit set depended on cross-pollination by bees, and increased with their frequent visit to flowers (KLEIN *et al*., 2003). In sunflower (*Helianthus annuus* L.), honeybees were the most frequent visitors and had the highest pollination efficiency index (Nderitu *et al*., 2008).

This study reports on the pollination system in *J. curcas*. The objectives of this work were to resolve the following questions: (i) what is the relative importance of cross-

and self-pollination for fruit set; (ii) do honeybees contribute effectively to fruit set; (iii) is *J. curcas* pollen-limited tautology; and (iv) do self-fertilised progeny of *J. curcas* display inbreeding depression?

5.2 MATERIALS AND METHODS

5.2.1 Study area

Experiments were conducted in a mono-culture plantation at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga) Pietermaritzburg, South Africa (30˚ 41' E, 29˚ 67' S; 781 m a.s.l). Pot experiments were conducted in a shade-house in the Botanical Garden of the University of KwaZulu-Natal, Pietermaritzburg, with average light photosynthetic photon flow density of 331 µmol m^{-2} s^{-1} at mid-day.

5.2.2 Study species

Physic nut, *Jatropha curcas* L. (Euphorbiaceae), is a small tropical tree or large shrub native to Mexico and Central America (HELLER, 1996). The plant is monoecious displaying protandry, and the flowers are unisexual. Occasionally, hermaphrodite flowers (**Figure 5.1A**) are present (DEHGAN and WEBSTER, 1979). In a previous study CHANG-WEI *et al*. (2007) reported that the male flowers (**Figure 5.1C**) opened first and a few flowers bloomed each day in each raceme (**Figure 5.1E**). A large number of female flowers (**Figure 5.1B**) opened from day-3 to day-5 after the male flower opened. In our study population, the mean number of male and female flowers per inflorescence (± S.E), calculated from 20 inflorescences, was 101.33 ± 4.49 and 6.44 ± 0.48, respectively. Thus, the male:female ratio was 16:1.

5.2.3 Breeding system

To examine the reproductive system of *J. curcas*, four pollination experiments were conducted on 38 plants between 18 December 2006 - 28 January 2007. Pollinators were excluded from all flowers by bagging the inflorescences with nylon mosquito-net bags (0.5 mm mesh) before anthesis. The treatments were: (i) Bagged flowers, 375

female flowers on 102 inflorescences were bagged and left without manipulation to develop inside the bags; (ii) Open-pollination, 302 female flowers on 98 inflorescences were left without bagging, offered to foraging insects and bagged after 72 h to avoid losses; (iii) Hand-self-pollination, 253 female flowers on 93 inflorescences were hand-pollinated using pollen from other inflorescences on the same plant; and (iv) Hand-cross-pollination, 306 female flowers on 104 inflorescences were cross-pollinated using pollen from other *J. curcas* plants. Flowers in the last two groups were bagged immediately after handling to avoid pollen contamination by insects.

5.2.4 Pollen limitation

To determine the extent of pollen limitation, four supplementary hand-pollination treatments were carried out, (i) Control-1, flowers were left open to determine seed production under natural conditions on the same plants; (ii) Control-2, flowers were left open to determine seed production under natural conditions on separate plants isolated distantly at least 10 m from the Control-1 plants; (iii) Outcrossing, flowers were extensively hand-pollinated with pollen collected from flowers of other plants to determine the potential for seed production through additional outcrossing; and (iv) Selfing, flowers were hand-pollinated with self-pollen (from male flowers in the same inflorescence or another on the same plant) to determine potential seed production through additional selfing. Flowers were bagged with mosquito-net bags before anthesis, and bagged again 5 d after treatment to avoid losses. Control-1, outcrossing and selfing treatments were applied to 20 plants each, selected at random in a 0.2 ha plot, while control-2 was applied to a further 20 plants in the same plot. Pollen limitation index (L) was calculated as:

$L = 1 - (Po/Ps)$, where,

Po was the average percentage fruit set in open-pollinated controls (Control-1 and Control-2), *Ps* was the percentage fruit set by plants that received additional cross-pollen. A value of $L = 0$ indicates no pollen limitation in the population under study (LARSON and BARRETT, 2000).

Fruit-set, -weight, -size, the number of seeds per fruit, and seed weight were evaluated. Fruit set was considered to be the percentage of flowers per plant that set fruit and was normalised by using angular transformation. SPSS® release 15 statistical software was used for data analysis (SPSS Inc., Chicago, USA). In the breeding system experiment, we deployed pair-sample t-tests to compare reproductive success in bagged *vs.* open-pollinated flowers, and in self- *vs.* cross-pollinated flowers. For the results of the pollen limitation experiment, one-way ANOVA was used and significant differences were assessed using Tukey's multiple comparisons at $P < 0.05$.

5.2.5 Pollinator effectiveness

Flower visiting-insects in the study were observed during January 2007, when the peak of flowering occurred. The visiting-insects observed in the study site were honeybees (*Apis mellifera*), wasps (*Bembecinus tridens*), and houseflies (*Musca domestica*). Honeybees were the most abundant visitors, while other insects were rare. Therefore only the honeybee data were considered in this study. Honeybee visits were observed for 30 min between 08.00 – 15.00 h each day for five sunny days. To test the effectiveness of honeybee visits for fruit set and fruit quality in *J. curcas*, visitation experiments were conducted involving three treatments on ten plants. (i) Single-visit, 90 female flowers in 20 inflorescences on ten plants were bagged at bud stage. When the flowers opened, the bags were removed and the flowers were exposed to a single visit by a honeybee, then bagged again to avoid further visits. (ii) Multiple-visits, 85 virgin female flowers in another 20 inflorescences on ten plants were left without bagging and offered to multiple visits by honeybees.

(iii) Control (no visit), 65 virgin female flowers on a further 20 inflorescences on ten plants were bagged and left without exposure to any visits by honeybees.

Fruit-set, -weight, -size, the number of seeds per fruit, and seed weights were evaluated. For statistical analysis of the data we used one-way ANOVA and Tukey's post hoc tests ($P < 0.05$) to compare differences between treatments.

5.2.5 Inbreeding depression

To determine whether there was any evidence for inbreeding depression in *J. curcas* hand-pollination experiments were conducted in which flowers were either cross- or self-pollinated. Cross-pollination was performed using outcross pollen from other plants within the same plot. Self-pollination was conducted using pollen from different inflorescences on the same plant. After pollination, all flowers were bagged and left to develop until fruit maturity, then harvested. Fruits were stored at 20℃ in brown paper bags for later examination. After 7 months, (February 2008), the seed coats were removed and each seed sowed in an individual pot containing sterile potting soil. The soil mixture in each plastic pot (20 cm) was compost: bark (chipped and decomposed Pine): LAN (limestone ammonium nitrate): 2:3:2 NPK (nitrogen, phosphorus, potassium) (4:1:0.1:0.1). After 2 d germination was recorded which was rapid (> 80% complete in 2 d). Thus variations in germination times were not analysed. The germination rate was determined as the percentage of seedlings produced from the total number of seeds from each maternal plant sown. In March 2008, 1 month after emergence, seedling weight, -length, stem width, the number of leaves, and root lengths in the selfed- and outcrossed-progeny were measured. The proportions of fruit set and seedling germination were angular transformed. The t-test was used to analyse the effect of type of cross on seedling characteristics. Inbreeding depression (δ) was calculated as outlined by HUSBAND and SCHEMSKE (1995).

$\delta = 1 - (ws/wo)$, where,

ws was the mean fitness of selfed progeny, and *wo* was the mean fitness of outcrossed progeny. Inbreeding depression was calculated as (*ws*/*wo*) -1 when trait values of the selfed plants exceeded those of outcrossed individuals (BUSH, 2005).

5.3 RESULTS

5.3.1 Breeding system

The average number of seeds per fruit produced by open-pollinated flowers was more than ten times higher than those of treatment-(i) (auto-bagged) ($P < 0.001$, **Figure 5.2D**). Open-pollinated flowers produced significantly larger and heavier fruits, more seed per fruit and heavier seeds when compared to the bagged flowers (**Figure 5.2B-E**). Cross-pollinated flowers produced significantly more fruit than manually self-pollinated flowers (**Figure 5.2A**). There were no significant differences between cross- and self-pollinated flowers in terms of fruit size, fruit weight, the number of seeds per fruit, and seed weight (**Figure 5.2B-E**).

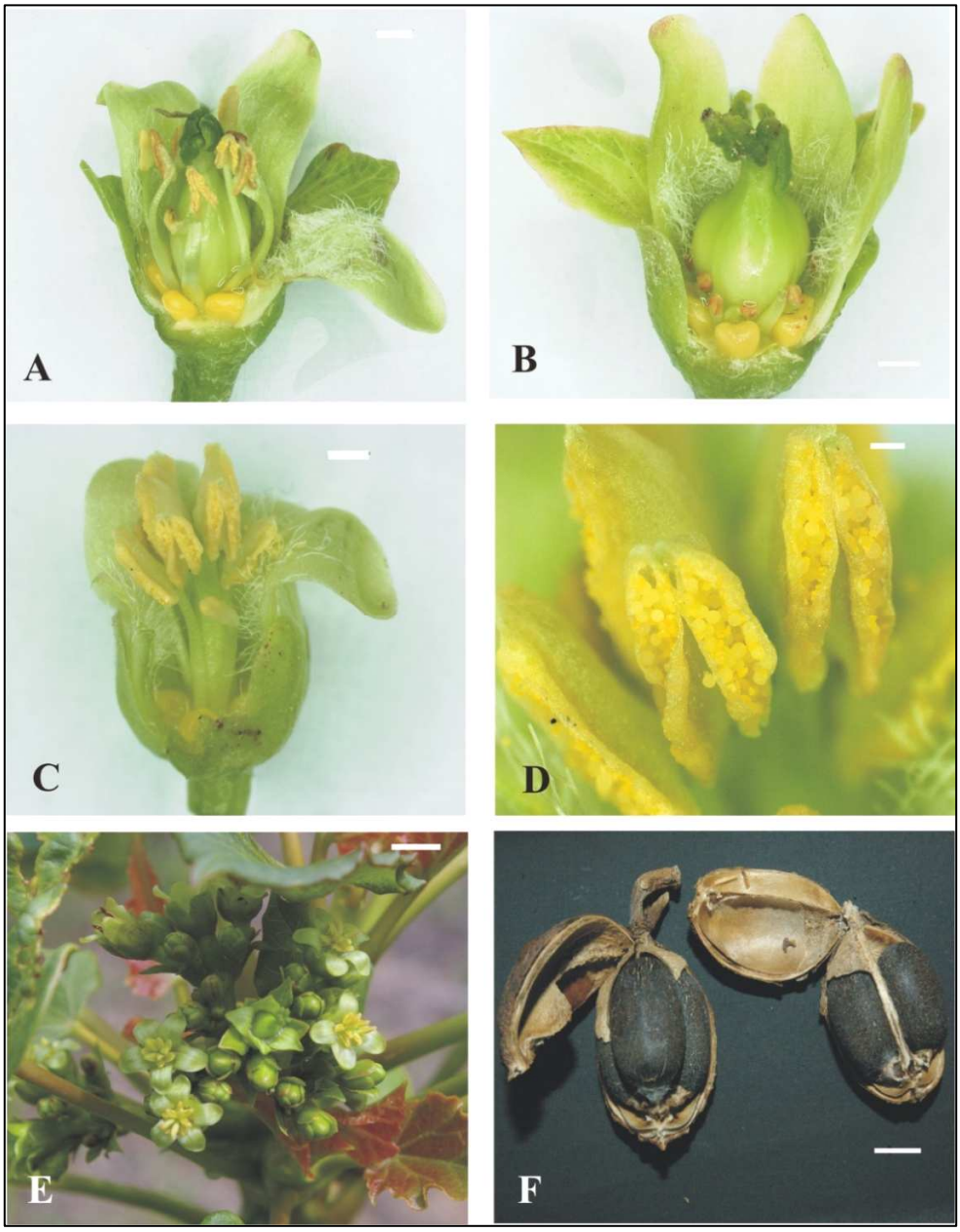

Figure 5.1 Flowers, floral organs and fruits of *Jatropha curcas*. (A) hermaphrodite flower; (B) female flower; (C) male flower; (D) anthers; (E) bisexual inflorescence; (F) fruits. Bar scale = 1 mm (A-D), 5 mm, (E) and 10 mm (F).

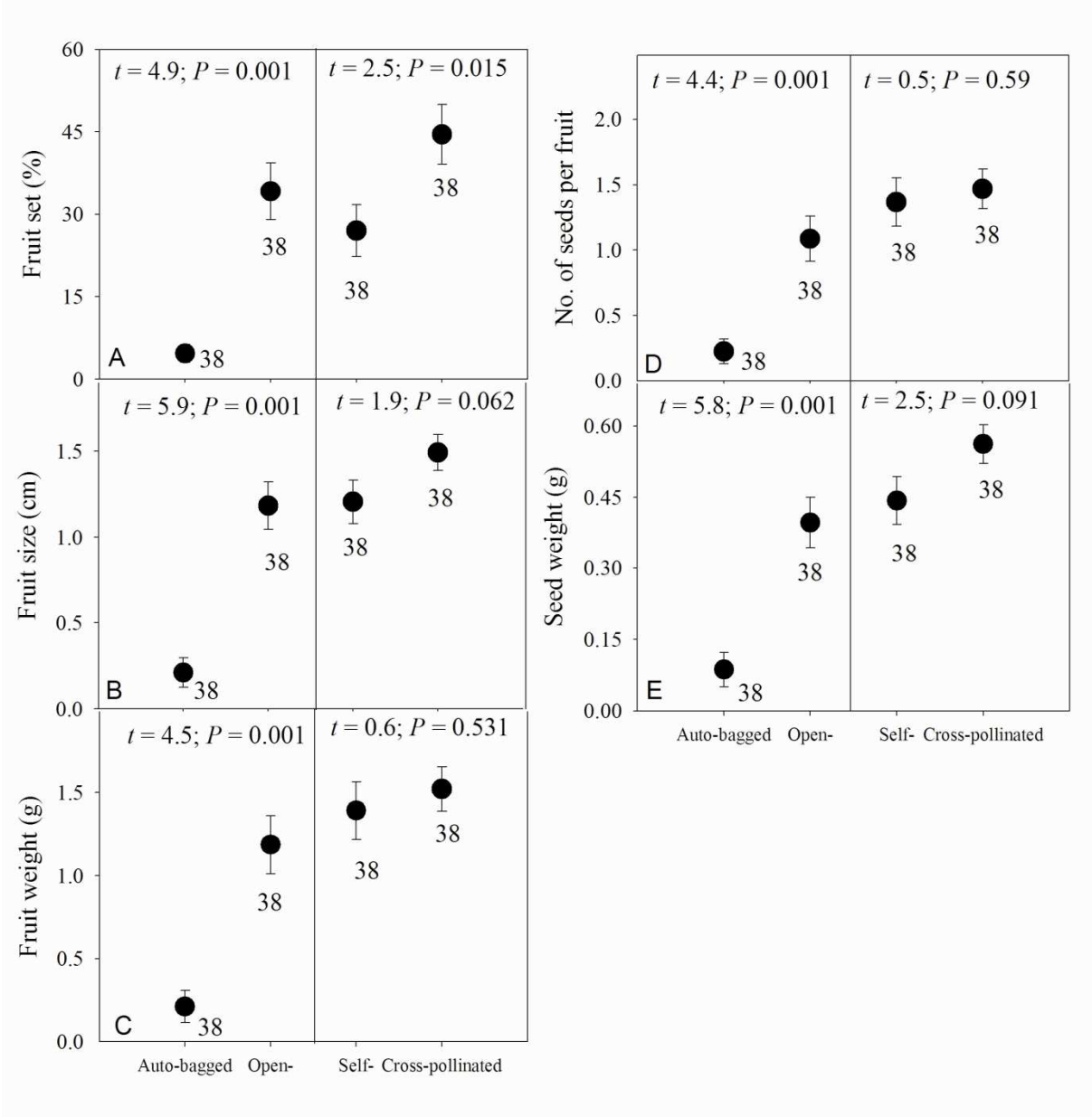

Figure 5.2 Breeding system experiment, comparison of fruit set percentage and fruit quality characteristics in auto-bagged (pollen excluded), open-pollinated (natural) and bagged (self-pollinated and cross-pollinated) flowers of *Jatropha curcas*. (A) fruit set, (B) fruit size, (C) fruit weight, (D) number of seeds per fruit, and (E) seed weight. Bars represent ± S.E. Sample size (number of plants) is given below each mean symbol.

5.3. 2 Pollen limitation

Supplemental hand-pollination did not significantly increase fruit set, fruit size, fruit weight, number of seeds per fruit, and seed weight relative to the control groups (**Figure 5.3**). The pollen limitation index was 0.03.

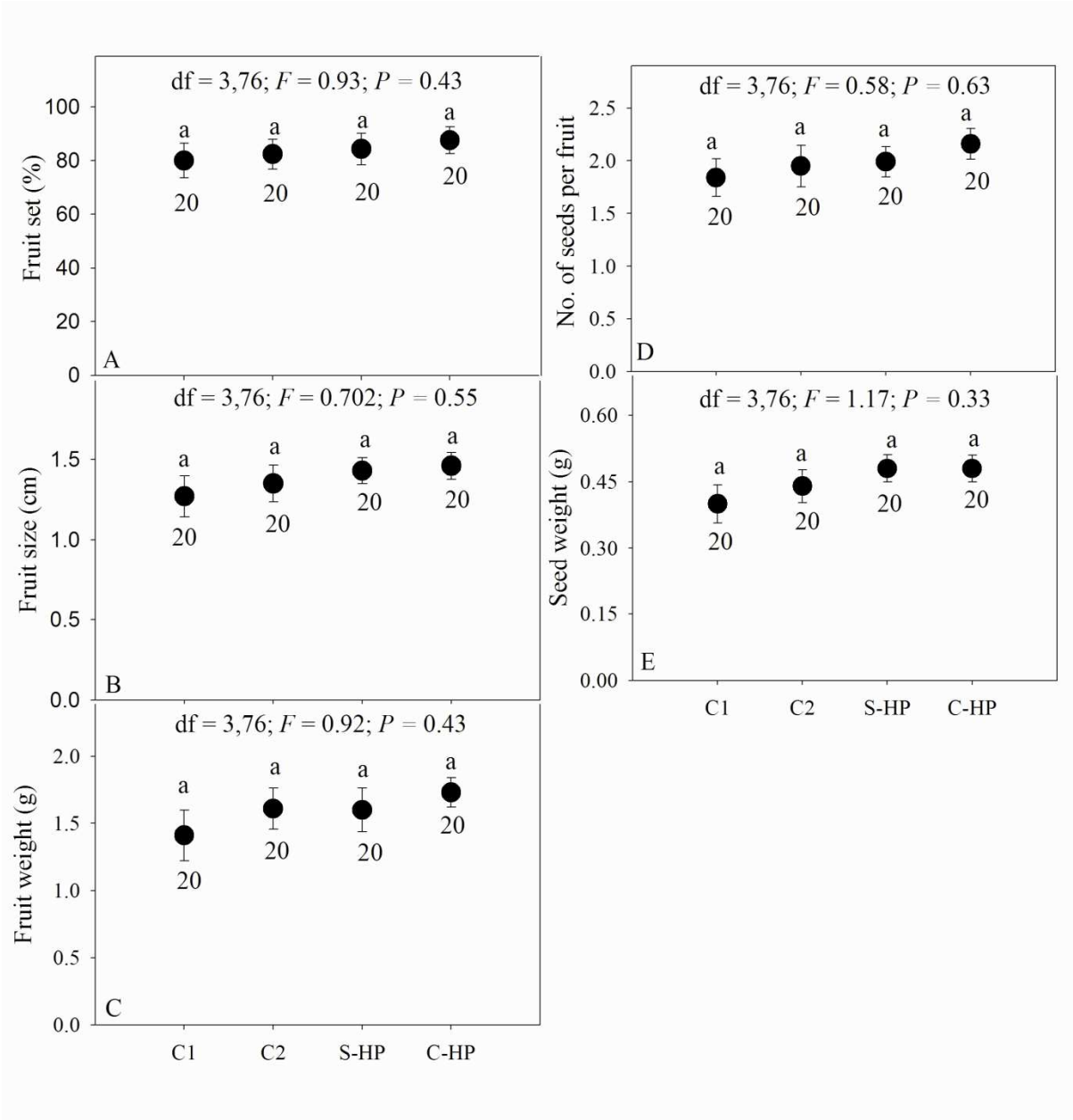

Figure 5.3 Pollen limitation experiment, fruit set and fruit quality measurements in *Jatropha curcas* flowers exposed to: natural pollination on the same plant Control (C1); natural pollination on different plants Control (C2); supplementary self-hand-pollination (S-H-P); or supplementary cross-hand-pollination (C-H-P). Bars represent ± S.E. Sample size (number of inflorescence) is given below each mean symbol.

5.2.3 Pollinator effectiveness

There were no significant differences in the durations of visits by honeybees on male or female flowers (means = 4.3 ± 0.25 s and 4.2 ± 0.26 s, respectively). The proportion of fruit produced by *J. curcas* flowers exposed to multiple or single visits by honeybees were significantly ($P < 0.001$) higher than those produced by flowers that received no visit (**Figure 5.4A**). Flowers exposed to multiple or single visits produced significantly larger and heavier fruits with many and heavier seeds than those produced by flowers from which honeybees were excluded. However, there were no significant differences between flowers exposed to a single or multiple visits by honeybees (**Figure 5C; Figure 5B-E**).

5.3.4 Inbreeding depression

Outcrossed flowers produced significantly more fruits than selfed-flowers (**Figure 5.5A**). Selfed-progeny had a significantly shorter root length compared to the outcross progeny (**Figure 5.5G**). However, there were no significant differences in mean fitnesses for selfed and outcross progeny for germination, seedling weight, stem length, stem width, and the number of leaves per seedling (**Figure 5.5C-F**).

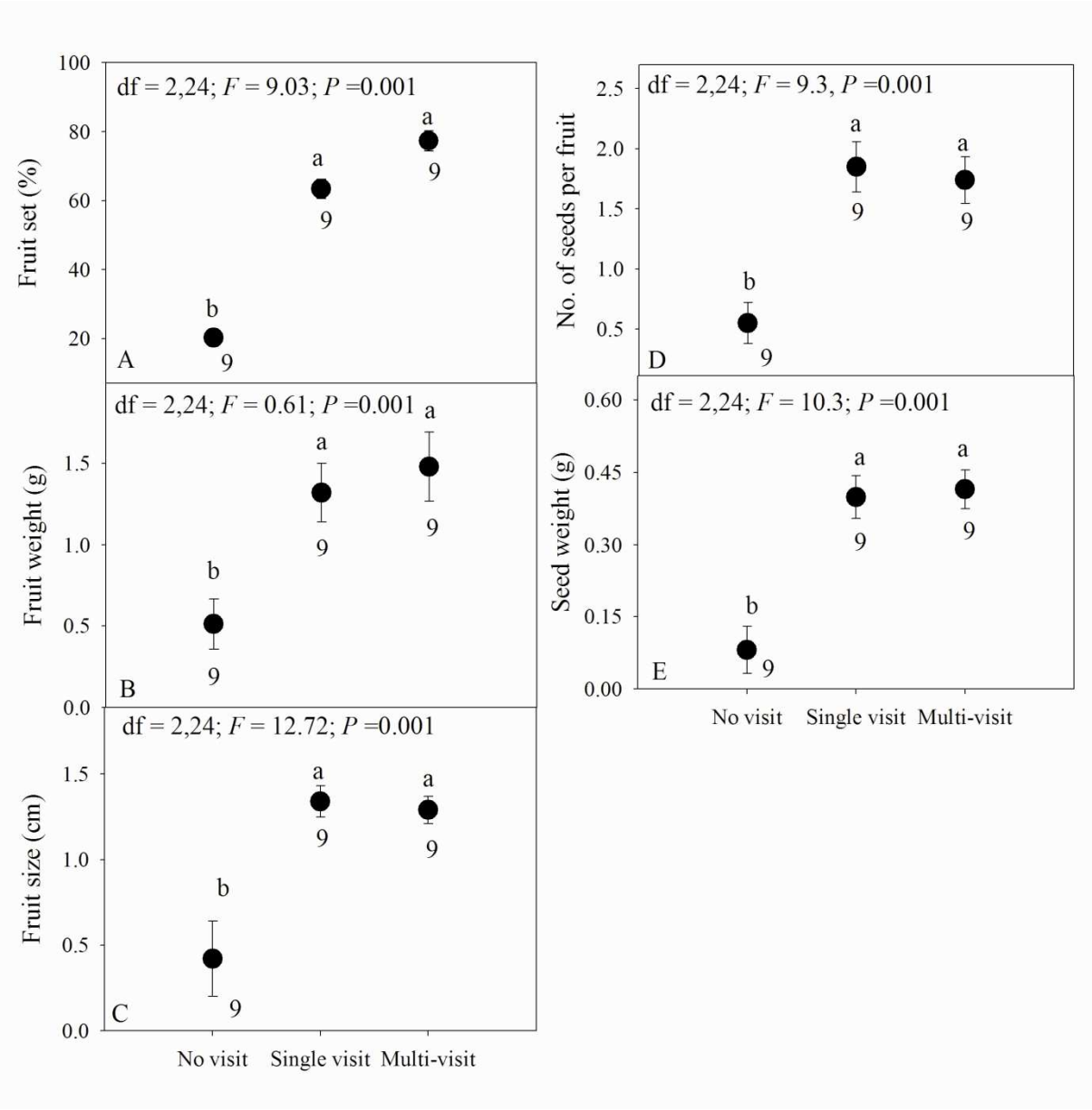

Figure 5.4 Pollinator effectiveness experiment, fruit set and fruit quality measurements in *Jatropha curcas* flowers that were bagged and/or exposed to a single or multiple honeybee visits. Bars represent ± S.E. Values with different letters are significantly different to each other according to Tukey's multiple range test at *P* < 0. 05. Sample size (number of flowers) is given below each mean symbol.

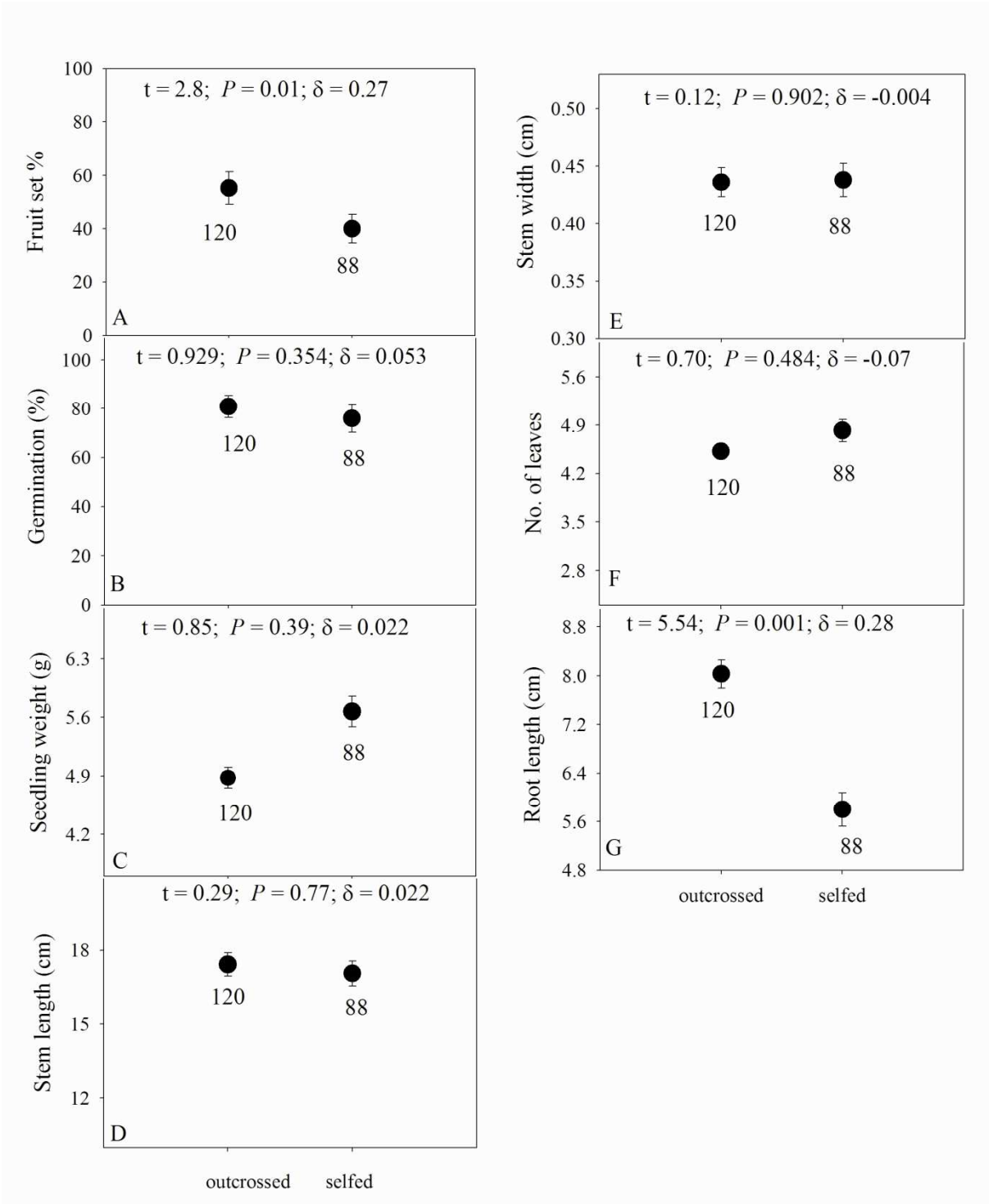

Figure 5.5 Inbreeding depression experiment, (A-G) mean fitness values for outcrossed- and selfed-progeny. (A) fruit set, (B) germination percentage, (C) seedling weight, (D) seedling length, (E) stem width, (F) number of leaves per seedling, and (G) root length. Inbreeding depression values (*δ*) were 1 – [*ws*/*wo*] when selfed-offspring had lower trait values than outcrossed-progeny, and [*wo*/*ws*] - 1 when trait values of selfed plants exceeded those of outcrossed individuals. Bars represent ± S.E. Sample size [number of seeds (A-B); number of seedlings (C-G)] is given below each mean symbol.

5.4 DISCUSSION

The breeding system results indicate that *J. curca*s is self-compatible, potentially reproducing through a mixture of self- and outcross-pollination, and rarely sets fruit as a result of autonomous selfing. However, 4% fruit set was obtained in the bagged flowers (**Figure 5.2A**). This could be due to wind pollination of the hermaphrodite flowers through the bag. Alternatively it could have arisen from low levels of apomixis in the female flowers (CHANG-WEI *et al*., 2007). Average seed production by open-pollinated flowers was higher than those of bagged flowers. Moreover, open-pollinated flowers produced significantly larger and heavier fruits with numerous and heavier seeds compared to the bagged flowers (**Figure 5.2B-E**). These findings are in line with those of DAG and GAZIT (2000) who reported that in mango (*Mangifera indica*) yield of small caged mango trees was miniscule (1 kg^{-1} tree), whereas open-pollinated trees carried a good crop (61 kg^{-1} tree). SHI and STÖSSER (2005) reported that in Chinese chestnut (*Castanea mollissima*) hand- and open-pollination resulted in fruit set levels of aproximately 90%.

In a previous study, honeybees foraged on both male and female flowers, making 64% of their total visits to male ones (RAJU and EZRADANAM, 2002). In another study, BHATTACHARYA *et al*. (2005) found that male flowers produced approximately 1617 viable pollen grains per flower, the pollen:ovule ratio was 539:1, and female flowers produce about 5 µl nectar, while male flowers produced approximately 2 µl nectar, the apomixis rate was 32%, and the seed:ovule ratio was 2:3. This nectar production by both types of flower was highly attractive to the honeybees. The results from this study showed that cross- and self- supplementary-hand-pollinated flowers did not significantly differ in fruit set and fruit quality characteristics compared to natural pollination in the two control treatments (**Figure 5.3A-E**). These results clearly indicate that *J. curcas* is not pollen-limited under the site conditions and during the specific time of year our experiments were conducted. Our explanation for these results is that the study site was a monoculture with a high density population that attracted a large number of bees. Plants occurring at low densities may suffer from insufficient pollen quantity by attracting fewer pollinators or receive fewer conspecific pollen grains per pollinator visit (FEINSINGER *et al*., 1986; KLINKHAMER and DE JONG, 1990). However, pollen limitation is often highly

variable among populations of a single species (DUDASH and FENSTER, 1997; BAKER *et al*., 2000; GOODWILLIE, 2001) and the possibility that other populations of *J. curcas* experience pollen limitation for fruit set cannot be excluded.

The results showed that flowers exposed to single or multiple visits by honeybees set a significantly ($P < 0.001$) higher proportion of fruit that were larger and heavier and with more numerous and heavier seeds than those which received no honeybee visits, indicating that honeybees were effective pollinators (**Figure 5.2 and Figure 5.4**). Similar results were reported for guava (*Psidium guajava*) by FREITAS and ALVES (2008) where a single honeybee visit to a flower produced significantly more fruit than non-visited flowers. Also, HOWPAGE and SPOONER-HART (2001) found that kiwifruit (*Actinidia deliciosa*) vines, which had no access to honeybees, had a significantly lower percentage fruit set (24%) compared to vines accessed by honeybees (91%). In oil seed rape (*Brassica campestris*), plants in plots visited by bees produced 58% more seeds and 46% larger seeds than those in plots from which bees were excluded (LANGRIDGE and GOODMAN, 1975). The results are similar to those of NDERITU *et al*. (2008) who found that sunflower (*Helianthus annuus*) plants in plots where insect visitors had access, produced on average, 53% more seed compared to plants in plots in which insect visitors were excluded. In *Coffea arabica,* visits by bees resulted in a 25% increase in fruit retention and seeds in these fruits were more than 25% heavier and developed faster (ROUBIK, 2002). In blueberries (*Vaccinium corymbosum*), honey-bee-mediated cross-pollination increased the mean number of fully developed seeds per fruit by 27.5% (LANG, 1991).

Differences in seed set between outcrossed and self-fertilized flowers can indicate inbreeding depression if fewer seeds are produced through self-fertilization (WASER and PRICE, 1983). The results showed that selfed flowers of the maternal plants produced significantly fewer fruits than the outcrossed flowers. Moreover, the selfed progeny had significantly shorter roots than the outcrossed progeny. However, there were no significant differences between selfed- and outcrossed progeny in the other traits scored for evidence of inbreeding depression. Similar results were reported for avocado (*Persea americana*) where flowering behaviour enhanced the opportunity

for cross- and self-pollination. Fruit from self pollination had a higher rate of abscission compared to those from cross pollination (DEGANI *et al*., 2003). The survival advantage of outcrossed fruit is probably related to the fact that selfed progeny have less vigorous embryos than outcrossed progeny due to inbreeding depression (DEGANI *et al*., 2003). In general, the present results provide little evidence of reduced yield through pollinator-mediated self-fertilization or inbreeding depression in selfed progeny in *J. curcas*. Furthermore, in another *in vivo* study test of pollen-pistil interactions following self- and cross-pollination, neither pollen germination nor pollen tube growth were inhibited or interfered with as a result of self-pollination treatments (**Chapter 6**). Therefore, type of pollination has no influence on the success of fertilization in this plant However, the expression of inbreeding depression is known to vary throughout the life cycle (CRNOKRAK and ROFF, 1999; KOELEWINJ *et al*., 1999), thus measurements over the whole life span are needed for a reliable indication of the degree of inbreeding depression (CHARLESWORTH and CHARLESWORTH, 1987).

6 Pollen viability, pollen germination and pollen tube growth in *Jatropha curcas*

6.1 INTRODUCTION

Seed production in angiosperms depends upon a sequence of steps, including pollen transfer to the stigma, pollen germination, pollen tube growth, ovule fertilization, seed development and finally seed maturation (CRUZAN, 1989). Effective pollination is a prerequisite for fruit**-** and seed-set and of vital importance to realize optimal yield. A thorough knowledge of pollen biology and its manipulation are required for any rational approach to increase crop productivity (SHIVANNA, 2003). The viability and morphological homogeneity related to pollen quality are useful for plant breeders, geneticists and growers (BOLAT and PIRLAK, 1999). Pollen viability has been evaluated by: (1) staining techniques; (2) *in vitro* and *in vivo* germination tests; or (3) analyzing final seed set. The choice of method depended on the crop or species (DAFNI and FIRMAGE, 2000; DAFNI *et al*., 2005). In many species pollen germination is dependent on the addition of key substrates such as calcium nitrate to the germination media (STEER and STEER, 1989). Pollen tube growth is one of the most essential phenomena in the life cycle of flowering plants (HEPLER *et al*., 2001). Opportunities for self pollen to compete with cross pollen for access to stigma surfaces, stylar tissues, or ovules are influenced by a variety of floral traits including dichogamy and floral display size (RAMSEY and VAUGHTON, 2000). In some plants, direct measurements of pollen tubes indicate that they grow slower or have higher rates of attrition following self- rather than cross-pollination (AIZEN *et al*., 1990). In *J. curcas* self-pollination resulted in significantly lower seed set than cross-pollination (**Chapter 5**). However, there is very limited information about pollen biology and post self- and cross-pollination processes in *J. curcas.*

In this study, pollen viability, pollen germination and pollen tube growth following cross- and self-pollination were investigated in *J. curcas* flowers. Five basic questions were addressed: (1) What is the viability level of pollen from male and hermaphrodite flowers?; (2) Which staining technique is reliable in testing *J. curcas* pollen viability?; (3) What is the optimum medium for *in vitro* pollen germination and pollen tube

growth?; (4) Does exogenous IAA play a role in enhancing pollen germination and pollen tube growth in this plant; and (5) Does self pollination inhibit or interfere with fertilization in *J. curcas*?

6.2 MATERIALS AND METHODS

6.2.1 Study species

Jatropha curcas is a perennial, deciduous shrub which produces flowers in racemose inflorescences (**Figure 6.1G**). Male and female flowers (**Figure 6.1F, 6.1E**, respectively) are produced in the same inflorescence and occasionally hermaphrodite flowers (**Figure 6.1D**) are present (DEHGAN and WEBSTER, 1979). The average male to female flower ratio in the study site was 16:1 (**Chapter 5**). Male flowers are salver-shaped, sepals and petals are five each, stamens are ten, diadelphous, arranged in two tiers of five each. The anthers (**Figure 6.1B**) are yellow, oval-shaped, five in number and are present at the villose flower base (RAJU and EZARDANAM, 2002; CHANG-WEI *et al*., 2007). The pollen grains (**Figure 6.1A**) are yellow, globular, inaperturate; the exine is semitectate and verrucate. Female flowers are relatively larger, the styles and stigmas (**Figure 6.1C**) are three each, and the latter are bifid. The ovary (**Figure 6.1C, H**) has three carpels, each with a single locule producing one ovule (**Figure 6.1I**). The floral base is villose and contains five yellow elliptical glands under the ovary.

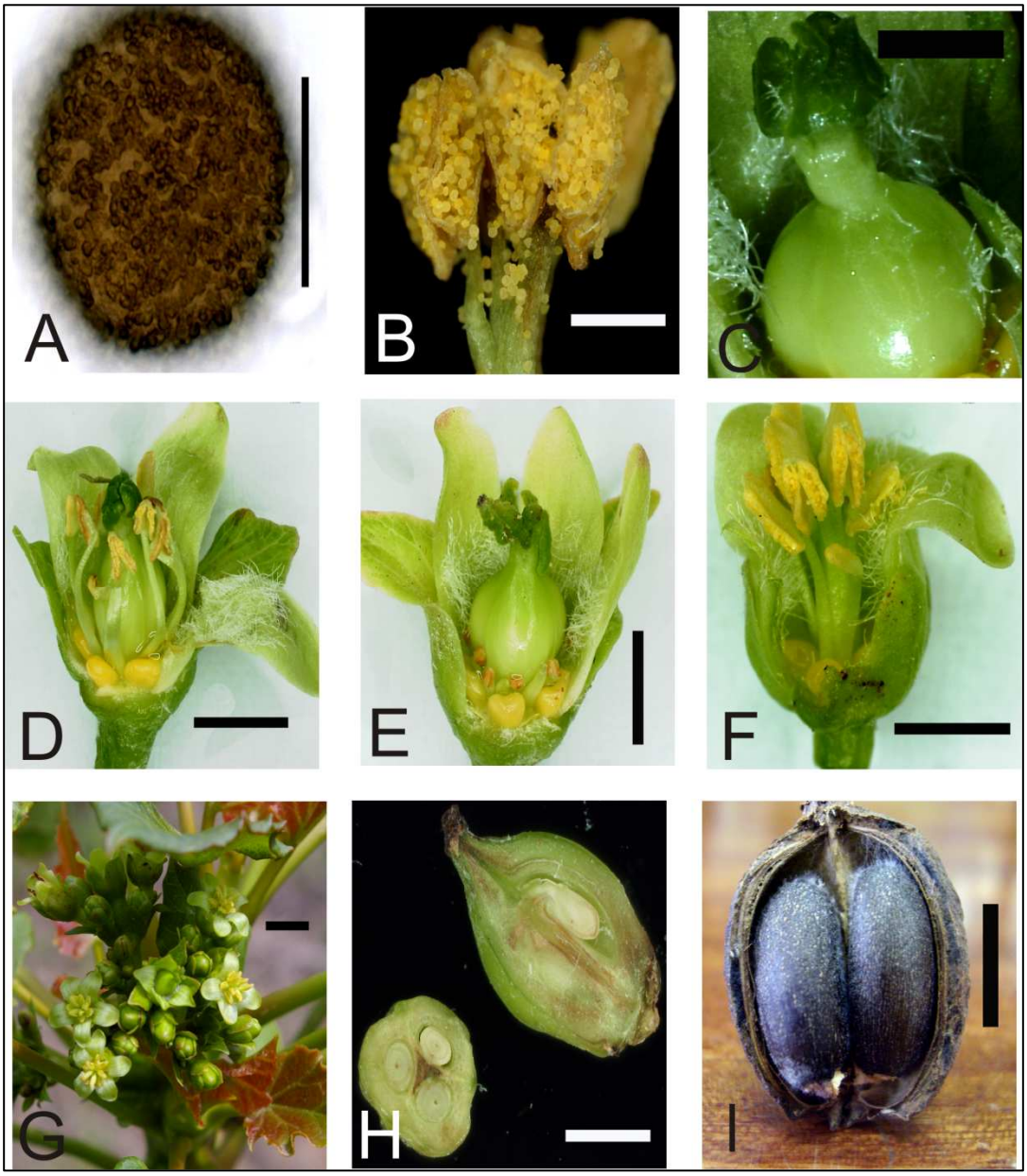

Figure 6.1 Pollen, floral organs, flowers, inflorescence, fruit and seeds of *Jatropha curcas*, (A) pollen; (B) anthers; (C) stigma and ovary; (D) hermaphrodite flower; (E) female flower (F); male flower; G) racemose inflorescence; (H) longitudinal and cross sections of ovary five days after pollination (DAP); (I) Fruit with two seeds. Bar scale = 1 mm (A-H) and = 1 cm (I).

6.2.2 Study site and plant materials

Branches with several inflorescences were collected from 50–60 trees in a monoculture plantation at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga), (Latitude 30˚ 41' E, Longitude 29˚ 67' S and Altitude 781 m above sea level), Pietermaritzburg, South Africa. The branches with inflorescences were taken to the laboratory and maintained at room temperature (25 ± 5˚C) and irradiance of 16 ± 2 µmol $m^{-2}s^{-1}$. They were placed in jars filled with tap water for three to four weeks until flowers opened and pollen grains were released.

6.2.3 Pollen viability tests

A number of staining methods were assessed for fresh and dead pollen. Pollen was killed by spreading a small amount of the pollen mixture into a 70 % ethanol droplet on a glass microscope slide, which was then heated with a flame and repeated two to three times (SHEFFIELD *et al*., 2005).

For testing for the presence of dehydrogenase, the test solution consisted of a 1% concentration of the substrate 2,3,5-triphenyl tetrazolium chloride (TTC) or 2,5-diphenyl monotetrazolium bromide (MTT) in 5% sucrose (KEARNS and INOUYE, 1993; KHATUN and FLOWERS, 1995; RODRIGUEZ-RIANO and DAFNI, 2000; ZENG-YU *et al*., 2004). The pollen grain was considered viable if it turned red in TTC and violet-purple in MTT (ZENG-YU *et al*., 2004; SHEFFIELD *et al*., 2005).

Aniline blue was used to detect callose in pollen walls and pollen tubes and the pollen grain was considered viable if it turned blue (KEARNS and INOUYE, 1993; KHATUN and FLOWERS, 1995; ZENG-YU *et al*., 2004). The aniline blue–lactophenol staining solution was prepared by adding 5 ml of 1% (w/v) aqueous aniline blue to a medium of 20 ml phenol, 20 ml lactic acid , 40 ml glycerol, and 20 ml distilled water (KEARNS and INOUYE, 1993).

For detecting starch content iodine and potassium iodide was used. Black-stained pollen was considered viable (KEARNS and INOUYE, 1993). For the fluorochromatic reaction (FCR) test for esterase activity and intactness of cell membranes, fluorescein diacetate was dissolved in acetone (2 mg/ml) and used at 10^{-6} mol l^{-1} in 0.8 mol l^{-1} sucrose (KEARNS and INOUYE, 1993; KHATUN and FLOWERS, 1995; ZENG-YU *et al*., 2004).

Pollen was viewed under Olympus AX70 Light- and Fluorescence-Microscopes. The total viable and non-viable pollen grains were counted in each field of view for a total count of at least 300 pollen grains. Staining percentage was determined by dividing the number of stained pollen grains per field of view by the total number of non-stained pollen per field of view and expressed as percentage after being normalized by using angular transformation. Non-viable pollen grains, which stay light coloured, were distinguished from viable ones. Data were analyzed using SPSS® version 15 (SPSS Inc., Chicago, USA) statistical software. Effects of treatments were analyzed using one-way analysis of variance (ANOVA). Tukey's test was used in order to compare the significance of differences among treatments.

6.2.4 *In vitro* pollen germination

Bulk fresh pollen grains were collected 2 days after anthesis, scattered uniformly into different liquid media, and incubated at 25˚C in darkness for 4 h. The different liquid media were: (1) control (distilled water); (2) control + [4 mg l^{-1} IAA]; (3) basal medium-1 comprised of 0.8 mmol l^{-1} sucrose + 0.7 mmol l^{-1} H_3BO_3 + 1.3 mmol l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ + 1% agar , hereafter referred to as M1, modified from ZENG-YU *et al*., (2004); (4) M1 + [4 mg l^{-1} IAA]; (5) basal medium-2 composed of 80 mg l^{-1} NH_4NO_3, 125 mg l^{-1} KNO_3, 125 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 125 mg l^{-1} KH_2PO_4, 50 mg l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 10 mg l^{-1} H_3BO_3, 3 mg l^{-1} $MnSO_4 \cdot 4H_2O$, 0.5 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.025 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 0.025 mg l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 50,000 mg l^{-1} sucrose, and 500 mg l^{-1} casein, hereafter referred to as M2, modified from JUANZI *et al*., (2008); and (6) M2 + [4 mg l^{-1} IAA].

Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain (TUINSTRA and WEDEL, 2000). A drop (2 µl) of a mixture of media and pollen were placed on a glass slide and covered with a cover slip. Germination percentage was determined by dividing the number of germinated pollen grains per field of view by the total number of pollen per field of view and expressed as percentage and normalized by using the angular transformation (KEARNS and INOUYE, 1993). Mean pollen tube length was calculated as the average length of 10 pollen tubes measured from each slide. For statistical analysis of data we used one way ANOVA analysis and Tukey's tests ($P < 0.05$) in order to compare differences between treatments.

6.2.5 *In vivo* pollen germination and pollen tube growth

To determine in vivo pollen tube growth, pollinations were conducted on the first day of flowering. Self-pollinations were made by brushing pollen from 2-4 anthers, taken from different flowers of the same inflorescence and plant, on the stigma. Cross-pollinations were similarly done by using pollen from other inflorescences from different plants. Hand pollinated flowers were collected at 2, 4, 6, 8, 24, 48, 72 and 96 HAP, longitudinally sliced and fixed in ethanol-acetic acid (3:1 v/v) for 24 h. After rinsing with water two to three times, pistils were cleared in 16% NaOH at room temperature for 3 days, or until most tissues became transparent. They were then rinsed in water and stained with 0.1% aniline blue in 0.1% K_2HPO_4 as outlined by KEARNS and INOUYE, (1993); TANGMITCHAROEN and OWENS, (1997).

Each half pistil was placed on a microscope slide with 10% glycerol and squashed under a glass cover slip. The number of pollen tubes and the rate of pollen tube growth in the style were examined using fluorescence microscopy. The differences in pollen tube length between self- and cross-pollinated flowers were compared by *t* test.

6.3 RESULTS

6.3.1 Pollen viability test

Tetrazolium salt (TTC) stained fresh pollen with a bright red colour (**Figure 6.2A**) and significantly ($P < 0.05$) distinguished between fresh and dead pollen compared to the other tests (**Table 6.1**). 2,5-diphenyl monotetrazolium bromide (MTT) however, did not differentiate between them (**Table 6.1**) and stained fresh and dead pollen deep violet-purple and orange-brown (**Figure 6.2B**). In the fluorchromatic test both fresh and dead pollen fluoresced, however, there were variations in the degree of fluorescence. Some pollen fluoresced very brightly, others less and others were non-fluorescent (**Figure 6.2E; Table 6.1**). Analine blue-lactophenol and IKI stained fresh and dead pollen with a dark blue and black colour respectively, thus, both tests did not adequately distinguish between fresh and dead pollen (**Figures 6.2C and 6.2D; Table 6.1**).

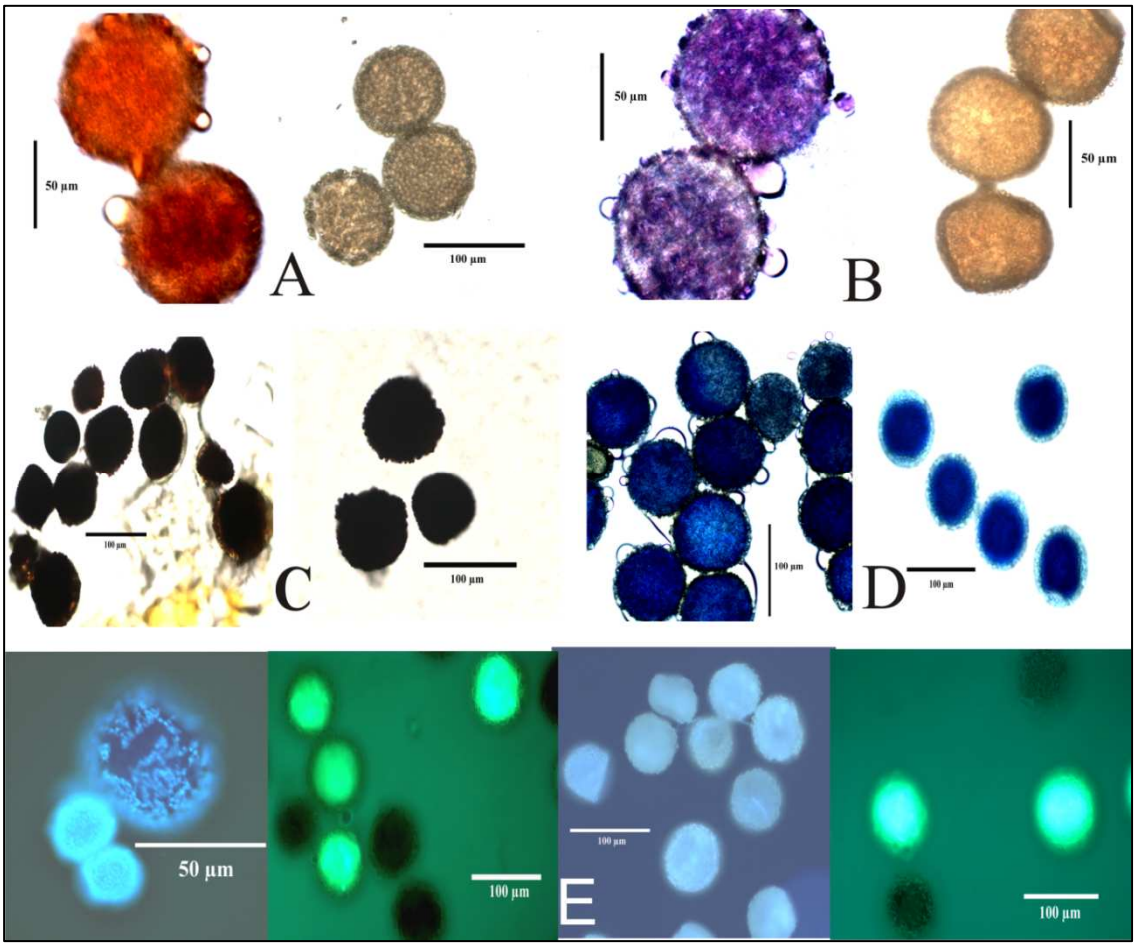

Figure 6.2 Response of *Jatropha curcas* fresh- and dead-pollen (left and right, repectively) to different staining tests, (A) TTC; (B) MTT; (C) IKI; (D) Analine blue-lactophenol; and (E) FDA.

Table 6.1 Percentage of stained fresh and dead pollen treated with TTC, MTT, IKI FDA or aniline blue. Data means ± S.E of five slides with two fields of view each represent a replicate (N = 10). A total of 300-400 pollen grains were counted per each field.

	Percentage of stained pollen (%)	
Test	**Fresh**	**Dead**
TTC	95.4 ±1.1^{ab}	22.3±3.8^{d}
MTT	92.1.8 ±2.3^{b}	91.3±1.49^{b}
FDA	76.4 ±1.8 ^{c}	71.0±6.4^{c}
IKI	97.0 ±1.5^{a}	97.8±1.6^{a}
Aniline blue-lactophenol	93.9 ±1.2^{b}	79.0±2.7^{c}

Mean ± S.E followed by the same letter(s) are not significantly different to each other at *P* < 0.005 according to Tukey's test.

6.3.2 *In vitro* pollen germination and pollen tube growth

6.3.2.1 Pollen germination

Pollen from male flowers germinated significantly ($P < 0.05$) better in M1 compared to the control and the other media (**Figure 6.4A**). However, supplementation of IAA to M1 and M2 significantly ($P < 0.05$) increased pollen germination compared to the control and control-plus-IAA treatments (**Figure 6.4A**). Pollen from hermaphrodite flowers had a lower germination rate compared to the male flowers and there were no significant differences in germination between all treatments (**Figure 6.4B**).

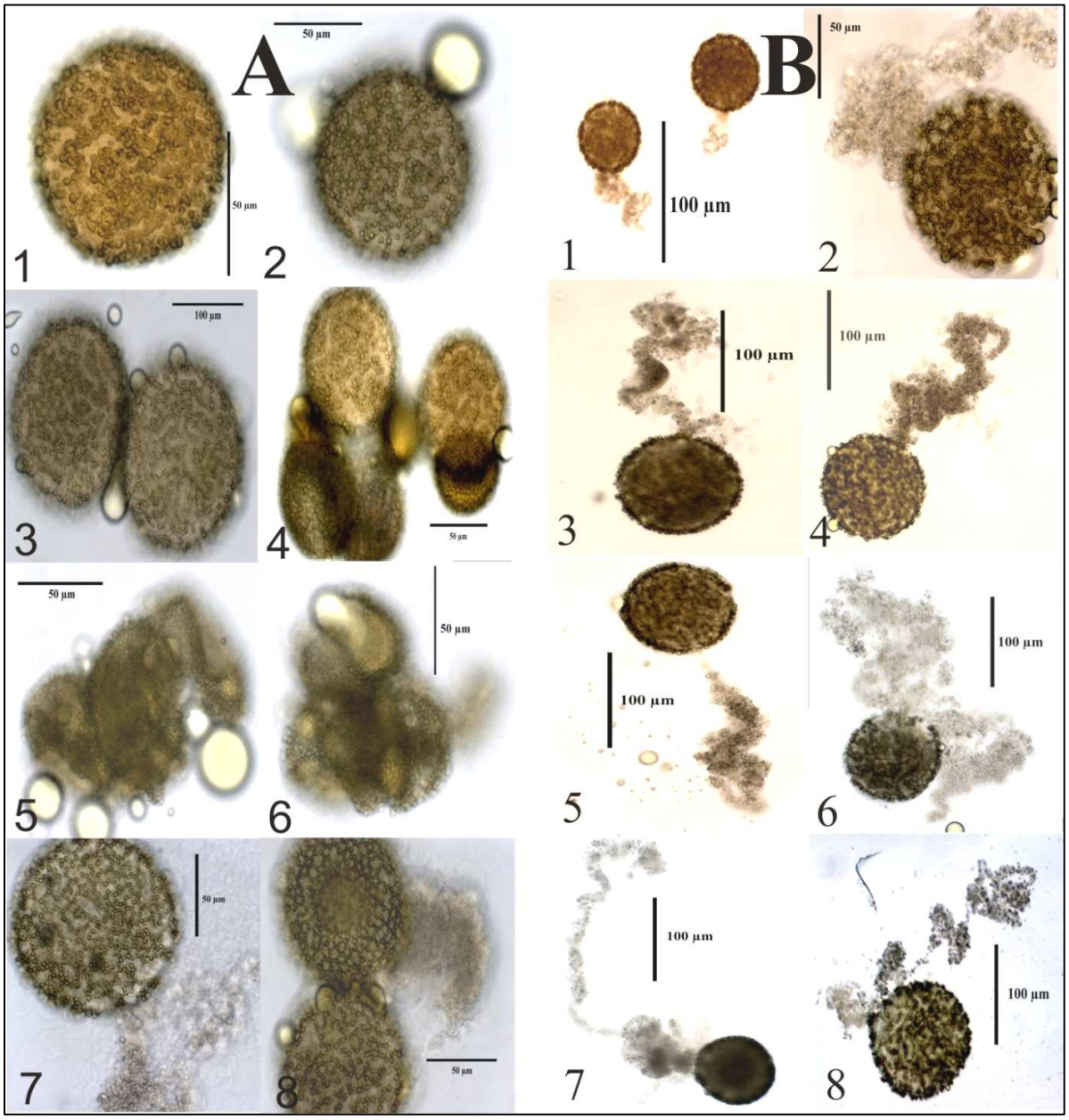

Figure 6.3 *In vitro* pollen germination and pollen tube growth of *Jatropha curcas*. (A) Hermaphrodite flower pollen: (A1) Non-germinated pollen grain; (A2 and A3) Pollen grains bursting as first sign of germination; (A4-A8) Abnormal pollen germination in response to different medium. (B) Male flower pollen: (B1-B2) Pollen tube from control treatment (distilled water); (B3-B4) Pollen tube growth in response to M2; (B5-B6) Pollen tube growth in response to M1+; (B7-B8) Pollen tube growth in response to M1.

6.3.2.2 Pollen tube growth

Pollen tube length from M1 and M1+ was significantly ($P < 0.05$) longer compared to all other treatments (**Figures 6.3B5 and 6.4C**). However, M2 produced significantly ($P < 0.05$) longer tubes compared to the control, control+ and M2+ treatments (**Figure 6.4C**). There were no significant differences between M2+ and C+. However, they were both significantly ($P < 0.05$) different from the control treatment (**Figure 6.4C**). Pollen from hermaphrodite flowers had shorter pollen tubes when compared to those from male flowers. There were no significant differences in pollen tube length between treatments (**Figure, 6.3D and 6.4A**).

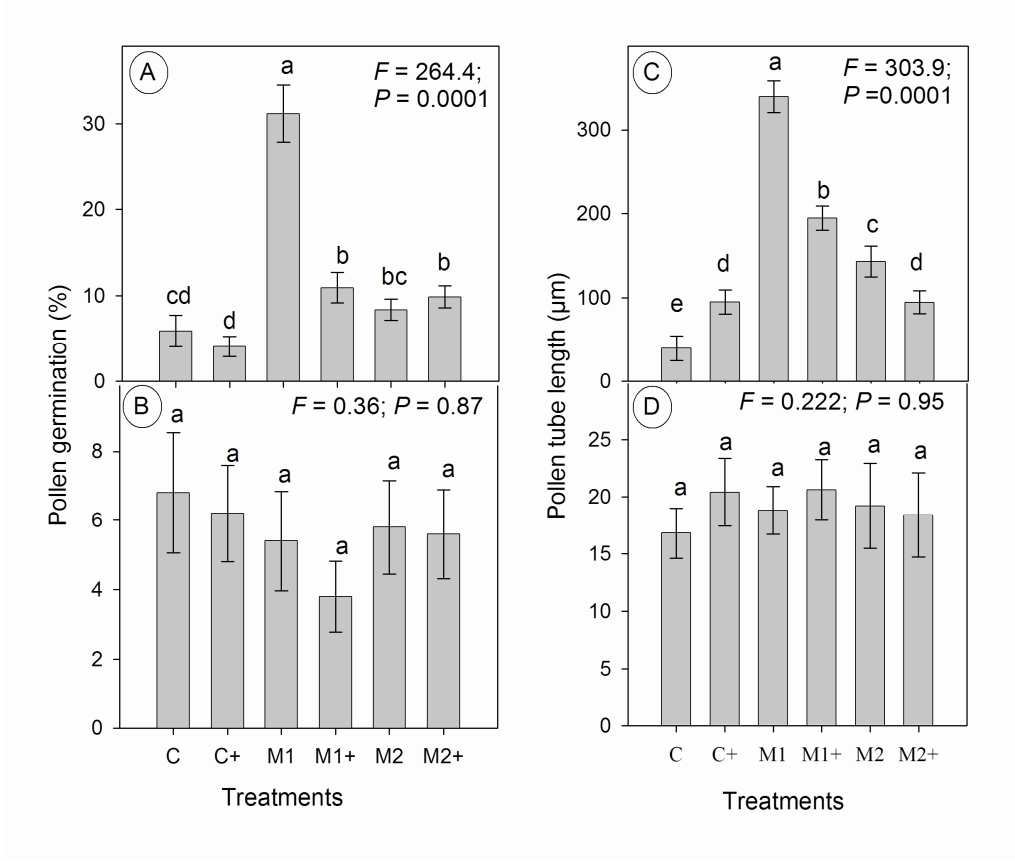

Figure 6.4: Mean *in vitro* germination percentage of *Jatropha curcas* (A) pollen from male flowers (B) pollen from hermaphrodite flowers in response to: C control, C+ (IAA + distilled water), M1 (basal medium-1), M1+ (basal medium-1 + IAA), M2 (basal medium-2), M2+ (basal medium-2 + IAA). Standard error (±) bars with different letter(s) are significantly different to each other according to Tukey's test (P < 0.05).

6.3.3 *In vivo* pollen germination and pollen tube growth

In both the self- and cross-pollination treatments a large number of pollen germinated on the stigmatic exudates. The pollen tubes formed callose plugs and elongated in the exudate in random directions (**Figures 6.5A1-2 and 6.5B1-2**). The number of pollen tubes reaching the base of the style was often substantially less than the number in the stigma (**Figures 6.5A3 and 6.5B3**). However, the average number of pollen tubes in the style was slightly higher in the cross- than in the self-pollination treatments (**Figure 6.6C**).

The average length of *J. curcas* styles in the study sample was 1300 ± 46.8 µm (**Figure, 6.1C**). Following self- and cross-pollination it took about 4 h for the pollen tubes tips to reach the middle of the styles, 6 h to teach the base of the style and they entered the ovary at about 8 h (**Figure 6.6A**). At 6 HAP the average pollen tube length and growth rate were significantly higher in the cross- compared with the self-pollinated flowers, 1380.5 ± 151.4 µm, 230.1 ± 25.2 µm h^{-1}, and 1137.8 ± 148.3 µm, 189.6 ± 25.3 µm h^{-1} respectively, (**Figure 6.6A and Figure6.6B**). There were no significant differences between 4 and 8 HAP. Nevertheless, cross- pollination pollen tubes attained greater length and a faster growth rate (**Figure 6.6A and 5.6B**).

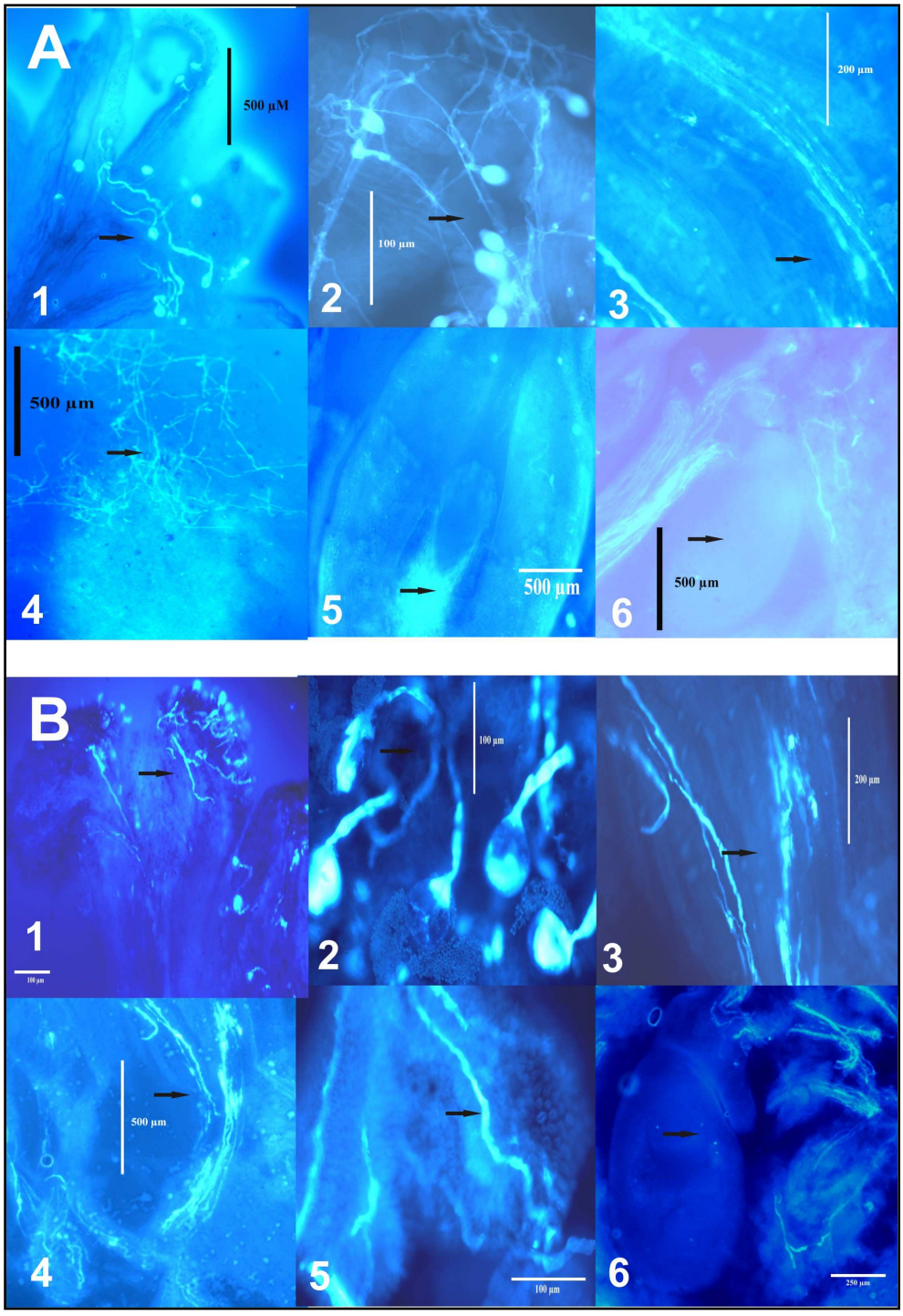

Figure 6.5 Fluorescence microscopy of *in vivo* pollen germination and pollen tube growth from (A) self-pollinated and (B) cross-pollinated *Jatropha curcas* flowers. In both A and B (Phases 1-5) are developmental stages within 0–8 h after pollination (HAP), (Phase1) pollen germination on the stigma, (Phases 2–3) a large number of pollen germinated on the stigma papillae and produced pollen tube that grew into the stylar canal (arrows), (Phase 4) pollen tube had entered the ovary, (Phase 5) pollen tube had penetrated the embryo, (Phase 6) developing embryo 72 HAP. Bar scale as shown in each panel.

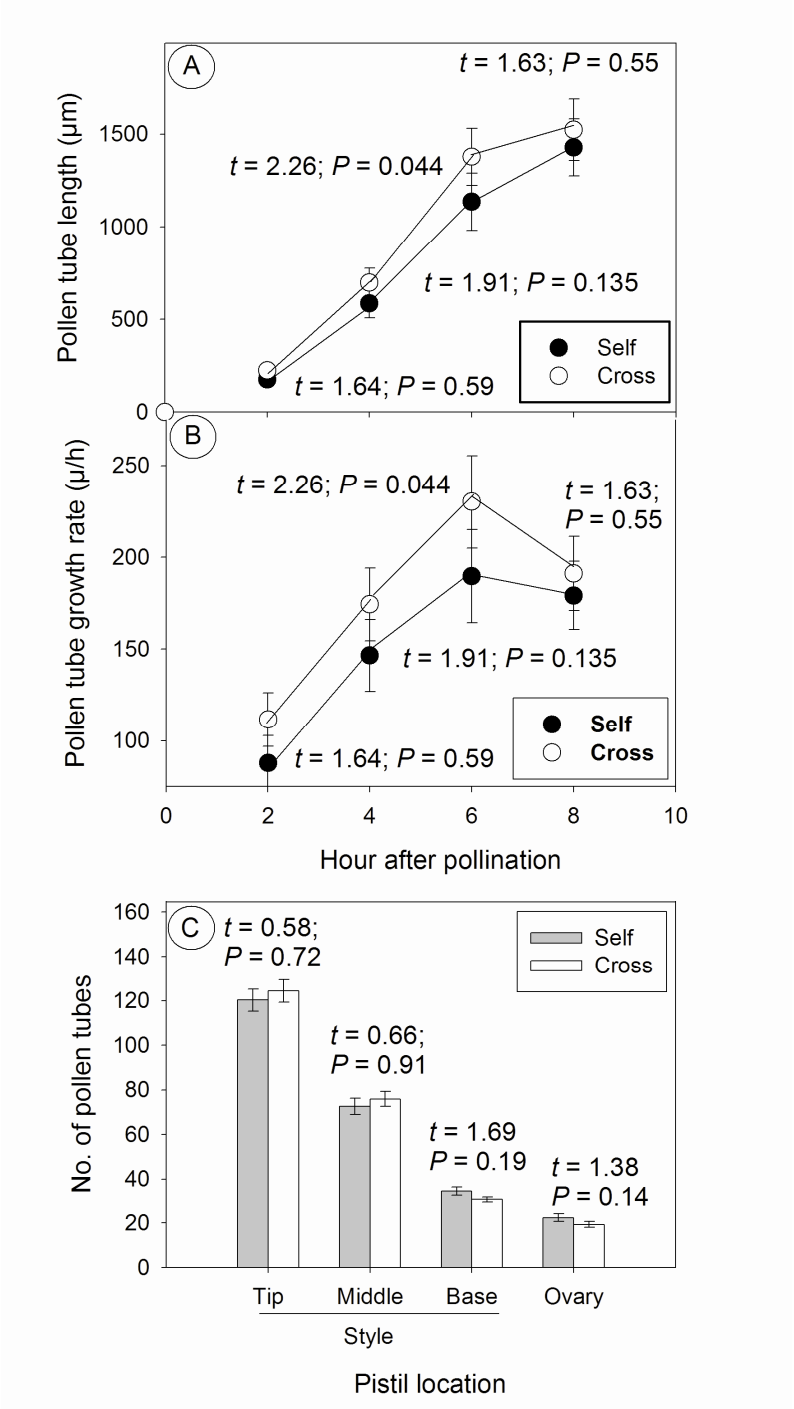

Figure 6.6 *In vivo* pollen tube growth following self- and cross-pollination of *Jatropha curcas* flowers, (A) pollen tube length 2, 4, 6 and 8 hours after pollination; (B) pollen tube growth rate 2, 4, 6 and 8 hours after pollination; (C) number of pollen tubes in tip, middle and base of the style and ovary. A–C values are means ± S.E (n = 10).

6.4 DISCUSSION

RODRIGUEZ-RIANO, and DAFNI (2000) recommended the use of heat-killed pollen as a control to check the potential of the dye for testing pollen viability. The study showed that tetrazolium salt TTC differentiated between fresh and dead pollen, thus the staining percentage of the fresh pollen (95% , **Table 6.1**) can be considered as the pollen viability percentage in the study sample (**Table 6.1; Figure 6.2A**). This is in line with HUANG *et al*., (2004) who reported that in (*Leymus chinensis*) no colour reaction was observed with dead pollen treated with TTC. However, MTT, FDA, IKI and blue aniline-lactophenol did not differentiate between fresh and dead pollen and therefore the staining percentage of the fresh pollen cannot be relied upon to determine pollen viability (**Figures 6.2B-E; Table 6.1**). These results, apart from those for TTC, agreed with ZENG-YU *et al*. (2004) who reported that in Tall fescue (*Festuca arundinacea*) TTC, MTT, FCR and aniline blue did not distinguish between fresh and dead pollen. Similar results were reported by PARFITT and GANESHAN, (1989) who found that in some *Prunus* species heat-killed pollen was intensely stained by MTT and HUANG *et al*., (2004) who found that in *Leymus chinensis* IKI stained the dead pollen in the same manner as fresh pollen.

Our results show that pollen from male flowers germinated well in M1 and M2 compared to the control (**Figure 6.4A**). These results are in line with TUINSTRA and WEDEL, (2000) who found that in Sorghum (*Sorgum bicolour*) germination was high in medium containing sucrose, boric acid and calcium nitrate. Furthermore, supplementation of IAA to M1 and M2 increased pollen germination compared to the control and control-plus-IAA treatments (**Figure 6.4A**). These results are in accordance with CHAUHAN and KATIYAR (1998) who found that IAA stimulated pollen tube growth in *Pinus kesiya*. Pollen tube length from M1 and M1-plus-IAA was longer compared to all treatments (**Figures 6.3B5 and 6.4C**). This is in line with JUANZI *et al*, (2008) who reported a distinct effect of exogenous IAA in *Torenia fournieri* L. which resulted in straighter and more slender pollen tubes compared with the controls. However, M2 produced longer tube length compared to the control, control-plus-IAA and M2-plus-IAA treatments (**Figure 6.4C**). There were no differences between M2-plus-IAA and control-plus-IAA in pollen tube length; however, pollen tube from both treatments was longer than the control one (**Figure**

6.4C). However, pollen from hermaphrodite flowers had a lower germination rate and shorter pollen tube length compared with that from male flowers and there were no differences in germination between all treatments (**Figures 6.3A and 6.4B, 6.4D**).

Our study shows that in both the self- and cross-pollination treatments, a large number of pollen grains germinated on the stigmatic exudates and formed callose plugs, indicating good growth of pollen tubes (**Figure 6.5A2 and 6.5-B2**). However, few of the pollen tubes were observed to elongate from the stigmatic exudates to the style. Similarly, FUSS and SEDGLEY (1991) found that in Scarlet Banksia (*Banksia coccinea*) pollen grains germinated on the stigma but very low numbers of pollen tubes grew down the style towards the ovary.

The average numbers of pollen tubes in the style was only slightly higher in the cross- than in the self-pollination treatments, indicating that there was no defect in the elongation of the pollen tube in the style (**Figure 6.6C**). These results are in line with OCKENDON and GATES (1975) who found that in *Brassica oleracea* the pattern of pollen tube growth was very much the same in the self- as in the cross-pollinated style. Similar results have been reported by SARR *et al*, (1983); HESSING, (1986); FENSTER and SORK, (1988). However, at 6 HAP, the average pollen tube length and growth rate was higher in the cross- compared to the self-pollinated flowers (**Figure 6.6A and 6.6B**). This finding is supported by AIZEN (1990) who found that in *Dianthus chinensis* average pollen tube length following self-pollination was shorter than the average pollen tube length following cross-pollination. However, he stated that higher growth rate of cross-pollen than self-pollen could not be explained by differences in pollination intensity or number of pollen grains germinated because the number of pollen tubes present in the selfed and crossed styles was similar. Also, CRUZAN, (1989); WELLER and ORNDUFF, (1989); LI *et al*., (2008) reported that in some plants, direct measurements indicate that pollen tubes grow slower or have higher rates of attrition following self- rather than cross-pollination.

Generally our study shows that neither pollen germination nor pollen tube growth were inhibited or interfered with self-pollination treatments. Both types of pollination

maintained their fertility as measured by penetration of an ovule by a pollen tube (**Figure 6.5-A5 and 6.5-B5**). This is also supported by the findings from **Chapter 5** on the breeding system of this plant that fruits arising from self-pollination are almost as numerous and large as those arising from cross-pollination.

7 Influence of plant growth regulators on flowering, fruiting, seed oil content, and oil quality of *Jatropha curcas*

7.1 INTRODUCTION

Knowledge of the influence of PGR-application on the flowering is of interest for both internal mechanisms regulating flowering and practical usefulness of controlling the time and degree of flowering (TOMPSEET, 1977). Abundant data indicate that use of PGRs may increase the yield of product per unit of time and land (MORGAN, 1980). In cottonseed, PGR application was reported to increase seed protein content, oil and protein yield ha^{-1}, seed oil refractive index, unsaponifiable matter and total unsaturated fatty acid content (oleic and linoleic acids) (SAWAN *et al*., 2001). *Jatropha curcas* oil contains about 14% free fatty acid (FFA), way beyond the limit of 1% FFA level that can be converted into biodiesel by trans-esterification using an alkaline catalyst (TIWARI *et al*., 2007). The fatty acid content reported in *J. curcas* oil by ADEBOWALE and ADEDIRE (2006) was 11.3% palmitic acid, 17% stearic acid, 4.7% arachidic acid, 12.8% oleic acid, and 47.3% linoleic acid. A significant increase in the seed hydrocarbon content in response to the application of PGRs in *J. curcas* was reported in a study by AGUSTUS *et al.* (2002). A means of obtaining improved flowering and fruiting in *J. curcas* would be of enormous commercial benefit. PGRs may eventually provide the means of bringing about such growth responses. However, the fruiting behaviour of *J. curcas* is bearing fruit bunches only at the apex of the branches. Therefore, limited branching is considered one of the major factors limiting yield in *J. curcas.* Traditionally MP (manual pruning) (**Chapter 3**) is practiced to promote branching in this plant. However, cost, convenience and efficiency of MP in large-scale plantations still remains a major concern. As mentioned in (**Chapter 4**) the following PGRs were used in this study as chemical pruners: BA (6-benzyladenine); TIBA (2,3,5-triiodobenzoic acid); Dikegulac (DK) (2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid); MH (Maleic hydrazide) (1,2-dihydro-3,6-pyridazinedione, coline salt). The results from (**Chapter 4**) have clearly established that *J. curcas* branching is very responsive to exogenous application of these PGRs.

It was suggested that the application of PGRs is often more consistently successful in enhancing flowering than cultural treatments (PHILIPSON, 1990). However, it is important to define the age of plants used in PGRs experiments, since the results can be influenced strongly by the stage of development at the time of treatment (ROSS, 1976). This study reports on the subsequent effects, after one year, following foliar application of these PGRs and MP on flowering, fruit set, fruit characteristics, seed total oil content, and FFA (free fatty acid content) in two-year-old plants of *J. curcas*.

7.2 MATERIALS AND METHODS

7.2.1 Study site and the experiment design

The experiment was conducted on one-year-old plants, at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga), Pietermaritzburg, South Africa (30˚41' E, 29˚67' S; and 781 m a.s.l). The plants were sprayed once in May 2007 and each plant received 200 ml of respective test solution. Manual pruning was done on the same day as that of the foliar treatment. The plants used in this experiment were all of the same variety with similar height and stem diameter. The foliar treatments consisted of BA (3, 6, 9, 12 and 15 mmol l^{-1}), TIBA (0.5, 1.0, 1.5 and 2 mmol l^{-1}), DK (2, 4, 6 and 8 mmol l^{-1}) and MH (2, 3 and 4 mmol l^{-1}). A small volume of sodium hydroxide (0.1 M) was used to solubilize PGRs before adding water. Plants sprayed with distilled water + an equivalent amount of 0.1 M NaOH served as control. A few drops of Tween® 20 (Merck) were added as surfactant. Each treatment consisted of twelve plants considering a single plant as one replicate selected randomly. In the subsequent year following the foliar spray (May 2008), data of the number of flowers per plant, fruit set percentage and the number of fruits per bunch were collected. In August 2008 fruits were harvested and fruit characteristics: number of fruits per plant, number of fruits per bunch, fruit- weight, -size, number of seeds per fruit and seed weight were measured. Fruit set was considered the percentage of flowers that set fruit per plant and was normalized by using angular transformation. SPSS® release 15 statistical software was used and one-way ANOVA was used for the data analysis (SPSS Inc., Chicago, USA).

7.2.2 Extraction of oil

The same seed samples used to determine the fruit characteristics were ground using an A11 BASIC analytical mill. Distilled *n-hexane* was used as solvent to extract the oil, in a Soxhlet apparatus (**Figure 7.1A)**. Three samples (3 g each) of the seed meal from each treatment were placed in Whatman single-thickness cellulose extraction thimbles. Empty round-base glass flasks were recorded for initial weight. Solvent (150 ml) was transferred to each flask and then placed on the Soxhlet plate. The Soxhlet system was connected firmly and ran for 2 h. The solvent was then removed from the extract using a Rotary Evaporator (Büchi) (**Figure 7.1B)**. The percentage of the extracted seed oil was determined using the equation:

$$\text{Oil content} = \frac{W_2 - W_1}{W_0} \times 100$$

Where, W_0 = the weight of the seed meal, W_1 = the weight of empty flask, W_2 = the weight of the flask with the oil extract.

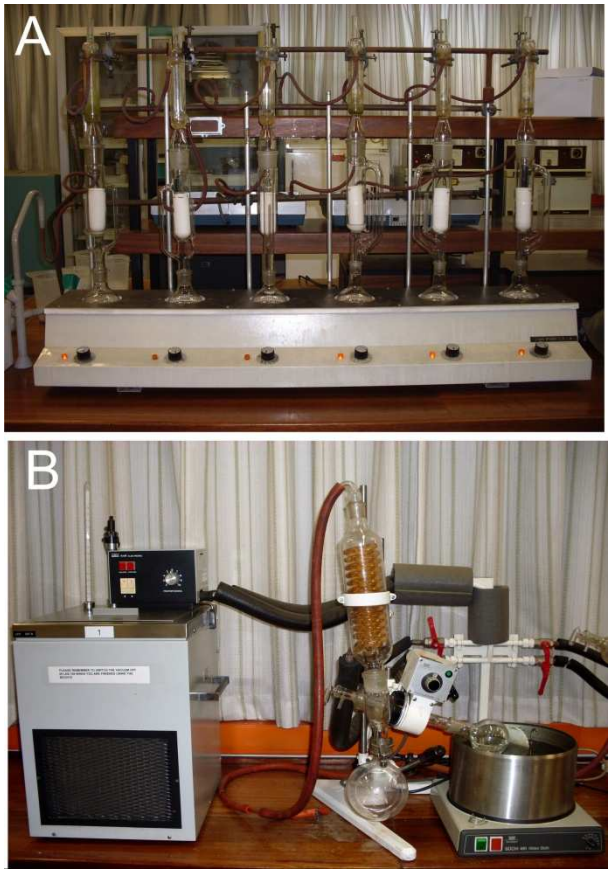

Figure 7.1 Oil extraction process, (A) Soxhlet apparatus; (B) Rotary Evaporator (Büchi-K4R) apparatus.

7.2.3 Oil analysis

Oil was analysed at the Department of Medicinal Chemistry, Faculty of Pharmaceutical Science University of Copenhagen, Denmark, as follows:

Oil (5 µl), 1000 µl MeOH (HPLC-grade), 200 µl (trimethylsily) diazomethane (Aldrich); were mixed and shaken for 15 min.

Glacial acetic acid (250 µl), 2.5 ml heptane (HPLC-grade), 3 ml saturated NaCl solution; were added, shaken for 30 min, and 1 ml of the heptane fraction was transferred into a GC-MS vial. The sample (1 µl) was injected into the GC-MS.

GC-MS

An Agilent 6890N Network GC system coupled to a 5973 Network Mass Selective Detector was used. GC conditions: injector temperature: 250 ℃; temperature

programme: start 70 ℃ hold for 4 min, 40 ℃/min to 160 ℃, 3 ℃/min to 270 ℃; column: HP5MS. Carrier gas: He.

7.3 RESULTS

7.3.1 Benzyladenine

Foliar application of BA at 3 mmol l^{-1} significantly ($P < 0.05$) increased the number of flowers per plant compared to the control and MP (manual pruning) (**Table 7.1**). BA at 12 mmol l^{-1} produced significantly ($P < 0.05$) more fruits per bunch compared to the control and MP. However, there were no significant differences in fruit set percentage (**Table 7.1**). BA at 9 mmol l^{-1} produced significantly ($P < 0.05$) heavier and bigger fruits compared to MP (**Figure 7.4A and B**). No significant differences were detected between treatments with respect to the number of seeds per fruit and seed weight (**Figure 7.4C and D)**. However, BA at 9 mmol l^{-1} produced a significantly ($P < 0.05$) higher seed oil content compared to MP.

7.3.2 Triiodobenzoic acid

TIBA at 1.5 and 2 mmol l^{-1} produced significantly more flowers per plant and more fruit per bunch respectively, compared to the control and manual pruning (**Table 7.2)**. However, no significant variations in fruit set percentage were found between treatments (**Table 7.2**). Foliar application of TIBA at all concentrations produced significantly ($P \leq 0.007$) heavier fruits compared to the control and MP treatments (**Figure 7.6A**). TIBA at all concentrations produced significantly ($P < 0.05$) fruits of bigger size and with more seeds per fruit compared to the MP treatments (**Figure 7.6B and C**). However, seed weight was not influenced by the different treatments (**Figure 7.6D**). TIBA at 1.5 and 2 mmol l^{-1} significantly produced seeds with higher oil content (**Figure 7.6E**). No significant differences in seed oil content were found between TIBA at higher concentrations (1.5 and 2 mmol l^{-1}) and the control treatment. However, TIBA at lower concentrations (0.5 and 1 mmol l^{-1}) reduced the seed oil content significantly compared to the control treatment (**Figure 7.6E**).

7.3.3 Dikegulac

DK at 2 mmol l^{-1} significantly increased the number of flowers per plant and the number of fruit per bunch compared to the control and MP treatments (**Table 7.3**). However, there were no differences between treatments in fruit set percentage (**Table 7.3**). Foliar application of DK at 2, 4, and 6 mmol l^{-1} produced significantly more seeds per fruit compared to the MP (**Figure 7.8D**). However, there were no significant differences between treatments in fruit weight, size and seed weight (**Figure 7.8 A, B and D**). DK at lower concentration (4 mmol l^{-1}) significantly produced higher seed oil content compared to the control and MP treatments (**Figure 7.8E**). At higher concentration (8 mmol l^{-1}), however, DK significantly reduced the seed oil content compared to the control (**Figure 7.8E**).

7.3.4 Maleic hydrazide

The number of flowers per plant was significantly increased by 1 mmol l^{-1} MH compared to the control treatment and MP (**Table 7.4**). MH at 2 mmol l^{-1} significantly ($P < 0.001$) produced heavier fruit compared to the control, MP and MH at higher concentration (4 mmol l^{-1}) (**Figure 7.10A**). Foliar application of MH at 1 and 2 mmol l^{-1} produced fruits significantly bigger in size, more seeds per fruit and heavier seeds compared to the control, MP and MH at higher concentration (4 mmol l^{-1}) (**Figure 7.10B, C and D**). MH at 2 mmol l^{-1} produced seeds with significantly higher oil content (**Figure 7.10A**).

7.3.5 Oil analysis

There were no variations in the FFA (free fatty acid contents) between the PGRs , MP and control treatments (**Table 7.5**). The average FAA content for the bulk sample were palmitic acid 18.2%, linoleic acid 41.7%, oleic acid 33.9% and stearic acid 6.1%. The highest FAA content for the different treatments were: 21.3% palmitic acid recorded for DK at 2 mmol l^{-1}, 48% linoleic acid recorded for TIBA at 0.5 mmol l^{-1}, 36.9% oleic acid recorded for BA at 3 mmol l^{-1} and 8.12% stearic recorded for MH at 2 mmol l^{-1} (**Table 7.5**).

Figure 7.2 *Jatropha curcas* fruits and seeds. (A) variation in fruit maturity; (B) variation in fruit size; (C) variation in number of seeds per fruit. Bar scale = 1 cm for (A-B), 0.5 cm for (C).

Table 7.1 Effects of BA, one year after foliar application, and MP (manual pruning), on the number of flowers/plant, fruit set percentage and the number of fruit per bunch in two-year-old plants of *Jatropha curcas.*

Concentration BA (mmol l^{-1})	No. of flowers/plant	Fruit set (%)	No. of fruit/bunch
0.00	53.7 ± 7.3b	86.7 ± 2.8	4.6 ± 0.7b
3.0	72.8 ± 4.7a	88.8 ± 5.4	2.7 ± 0.3c
6.0	30.0 ± 7.0c	81.6 ± 4.1	2.8 ± 0.4c
9.0	38.6 ± 6.3c	80.9 ± 2.9	4.0 ± 0.4bc
12.0	45.0 ± 9.2bc	80.5 ± 2.9	5.2 ± 0.7a
MP	38.8 ± 8.5b	84.6 ± 4.6	3.3 ± 2.7b

Means ± (S.E) followed by the same letter(s) are not significantly different to each other according to Tukey's test at P < 0.05.

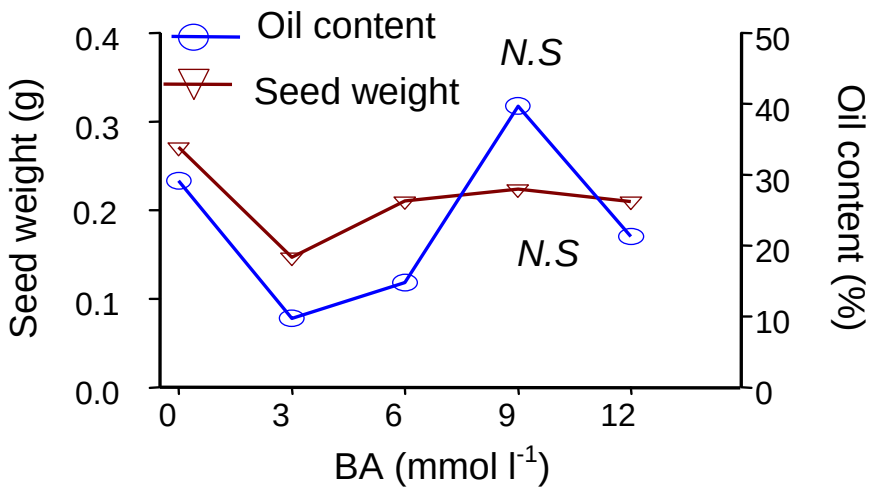

Figure 7.3 Seed weight and seed total oil content of two-year-old *Jatropha curcas* plants, one year after foliar application of BA and MP (manual pruning). *N.S* ≡ not significant according to Tukey's test at *P < 0.05*.

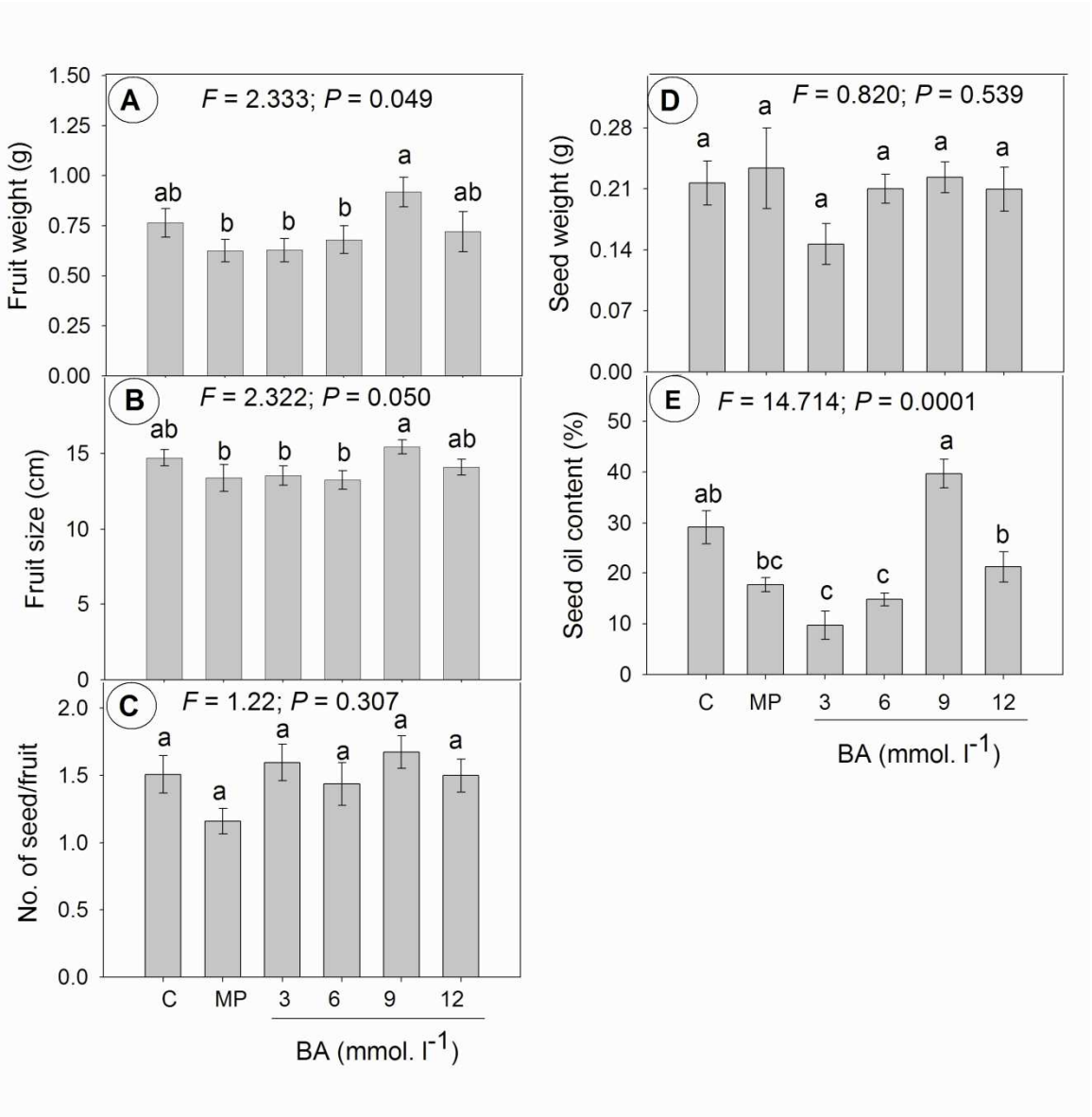

Figure 7.4 Effect of BA, one year after foliar application and MP (manual pruning), on fruit characteristics of two-year-old plants of *Jatropha curcas*. (A) fruit weight; (B); fruit size; (C) number of seeds per fruit; (D) seed weight; and (E) seed total oil content (%). S.E. bars sharing the same letter(s) are not significantly different to each other according to Tukey's test at *P* < *0.05*.

Table 7.2 Effects of TIBA, one year after foliar application, and MP (manual pruning), on the number of flowers/plant, fruit set percentage and the number of fruit per bunch in two-year-old plants of *Jatropha curcas.*

Concentration TIBA (mmol l^{-1})	No. of flowers/plant	Fruit set (%)	No. of fruit/bunch
0.00	53.7 ± 7.3b	86.7 ± 2.8	4.6 ± 0.7b
0.5	42.1 ± 6.3bc	77.4 ± 3.4	3.3 ± 0.7b
1.0	46.1 ± 5.3b	78.3 ± 3.2	3.3 ± 0.6b
1.5	69.6 ± 9.3a	78.2 ± 3.1	3.7 ± 0.5b
2.0	60.86 ± 5.1ab	77.9 ± 2.7	5.5 ± 0.9a
MP	38.8 ± 8.5b	84.6 ± 4.6	3.3 ± 2.7b

Means ± S.E. followed the same letter(s) are not significantly different to each other according to Tukey's test at P < 0.05.

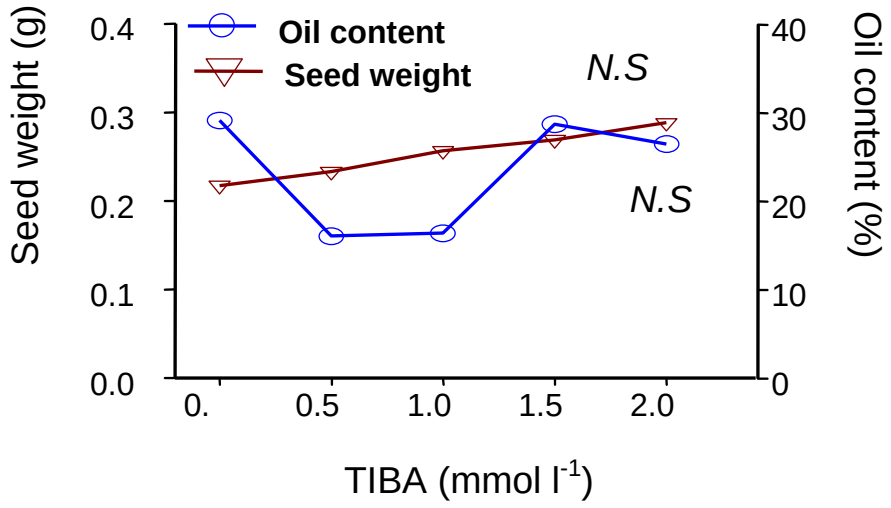

Figure 7.5 Seed weight and seed total oil content of two-year-old *Jatropha curcas* plants, one year after foliar application of TIBA and MP (manual pruning). *N.S* ≡ not significant according to Tukey's test at *P < 0.05*.

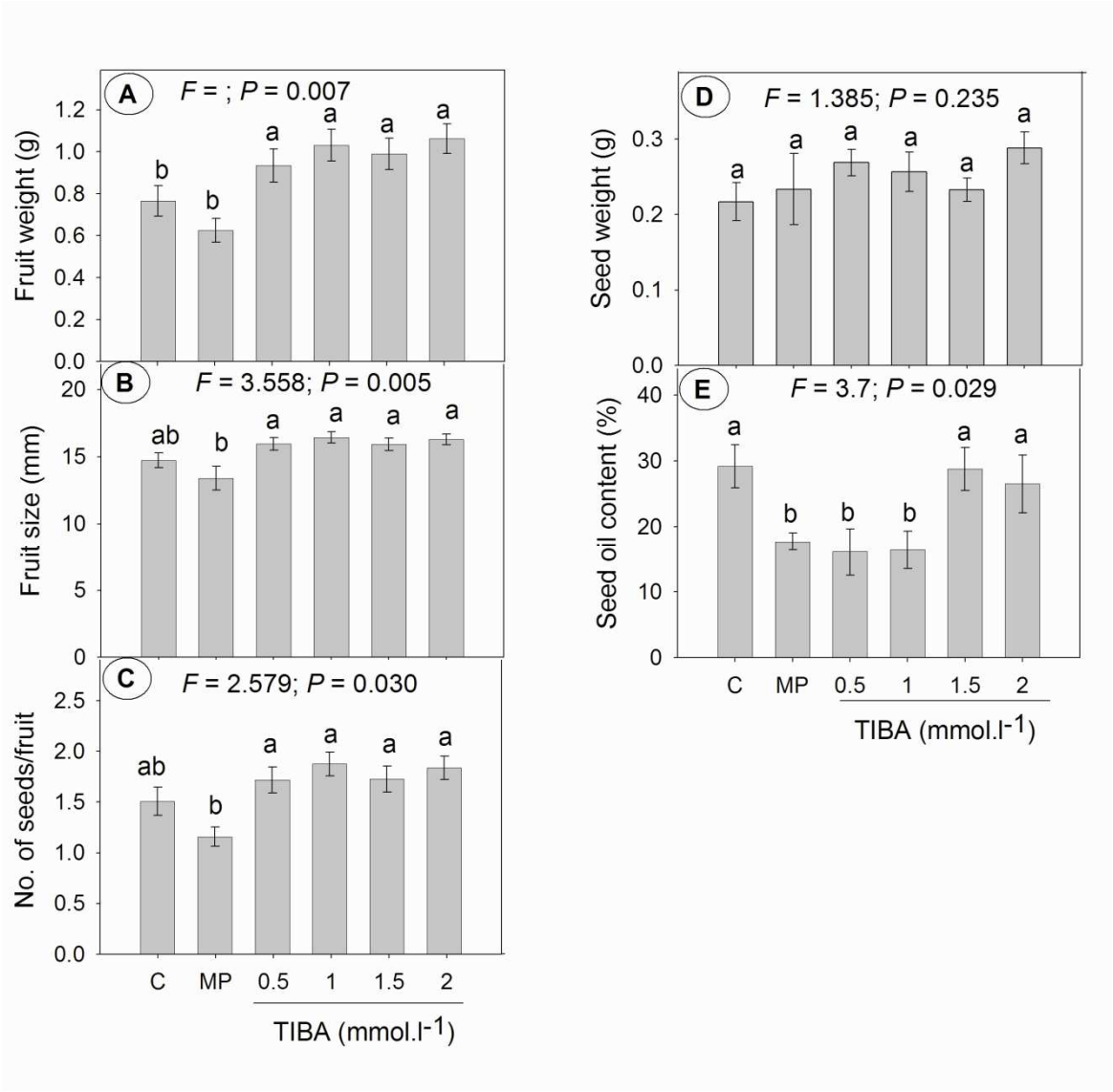

Figure 7.6 Effects of TIBA, one year after foliar application and MP (manual pruning), on fruit characteristics of two-year-old plants of *Jatropha curcas*. (A) fruit weight; (B); fruit size; (C) number of seeds per fruit; (D) seed weight; and (E) seed total oil content (%). S.E. bars sharing the same letter(s) are not significantly different to each other according to Tukey's test at *P* < *0.05*.

Table 7.3 Effects of Dikegulac one year after foliar application, and MP (manual pruning), on the number of flowers/plant, fruit set percentage and the number of fruit per bunch in two-year-old plants of *Jatropha curcas.*

Concentration DK (mmol l^{-1})	No. of flowers/plant	Fruit set (%)	No. of fruit/bunch
0.00	53.7 ± 7.3b	86.7 ± 2.8	4.6 ± 0.7b
2.0	71.25 ± 6.6a	76.4 ± 3.1	5.4 ± 0.6a
4.0	58.7 ± 4.9b	79.4 ± 4.6	3.1 ± 0.5b
6.0	51.4 ± 5.3b	71.3 ± 3.1	3.7 ± 0.9b
8.0	53.7 ± 8.2b	74.5 ± 7.6	4.0 ± 0.3b
MP	38.8 ± 8.5b	84.6 ± 4.6	3.3 ± 2.7

Means ± (S.E) followed by the same letter(s) are not significantly different to each other according to Tukey's test at P < 0.05.

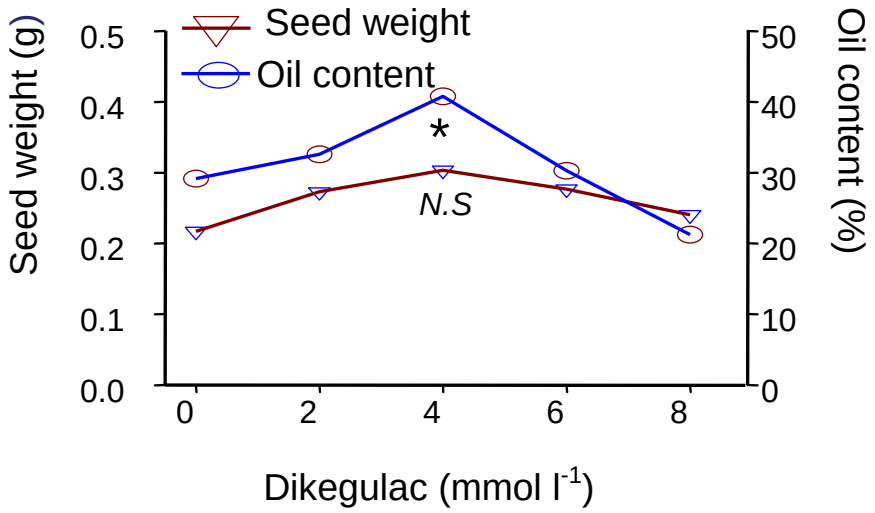

Figure 7.7 Seed weight and seed total oil content of two-year-old *Jatropha curcas* plants, one year after foliar application of Dikegulac and MP (manual pruning). * ≡ significant, *N.S* ≡ not significant according to Tukey's test at *P < 0.05*.

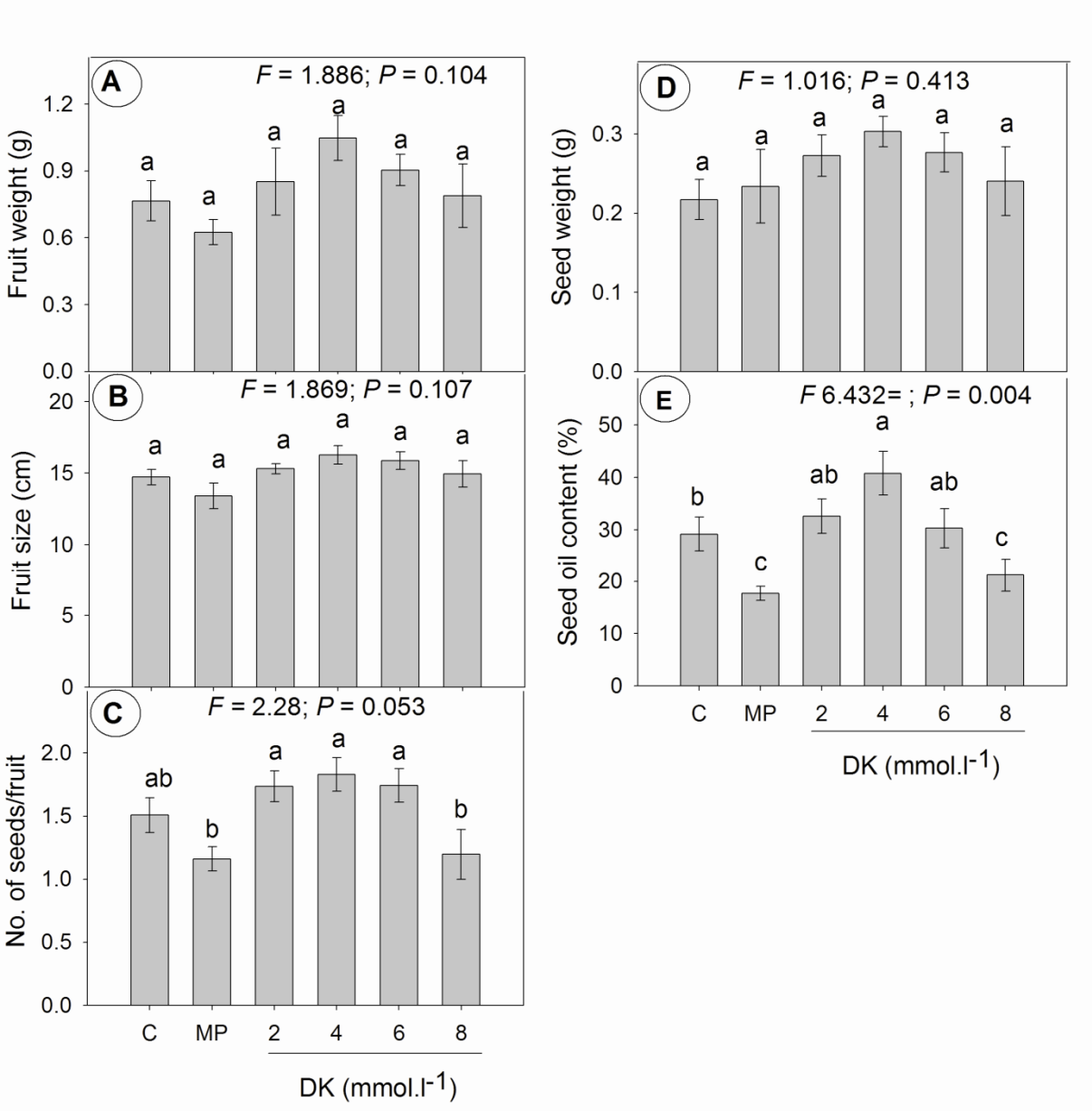

Figure 7.8 Effect of Dikegulac, one year after foliar application and MP (manual pruning), on fruit characteristics of two-year-old plants of *Jatropha curcas*. (A) fruit weight; (B); fruit size; (C) number of seeds per fruit; (D) seed weight; and (E) seed total oil content (%). S.E. Bars sharing the same letter(s) are not significantly different from each other according to Tukey's test at *P < 0.05*.

Table, 7.4 Effect of MH (Maleic hydrazide), one year after foliar application, and MP (manual pruning), on the number of flowers/plant, fruit set percentage and the number of fruit per bunch in two-year-old plants of *Jatropha curcas*.

Concentration MH (mmol l^{-1})	No. of flowers/plant	Fruit set (%)	No. of fruit/bunch
0.00	53.7 ± 7.3b	86.7 ± 2.8	4.6 ± 0.7
1.0	59.9 ± 10.2a	80.5 ± 3.5	2.9 ± 0.4
2.0	44.6 ± 4.9b	81.3 ± 4.2	3.6 ± 0.5
4.0	34.5 ± 3.9b	83.5 ± 3.9	4.8 ± 1.0
MP	38.8 ± 8.5b	84.6 ± 4.6	3.3 ± 2.7

Means ±(S.E) followed by the same letter(s) are not significantly different to each other according to Tukey's test at P < 0.05.

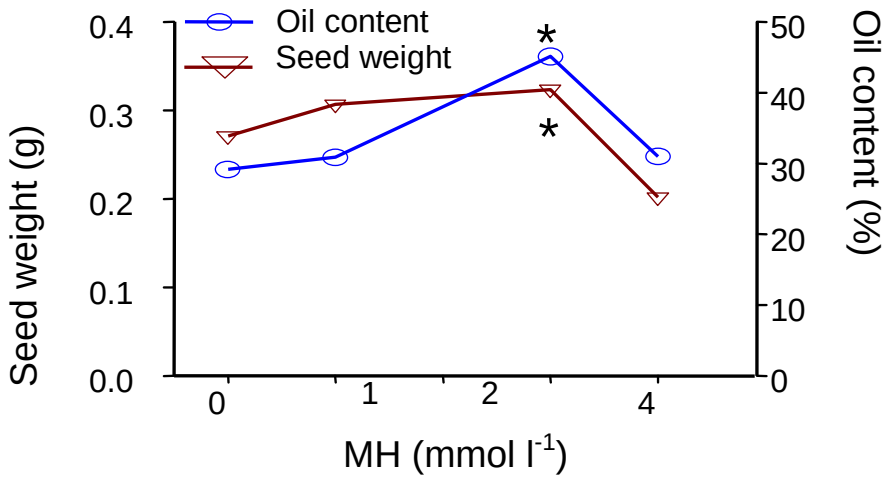

Figure 7.9 Seed weight and seed total oil content of two-year-old *Jatropha curcas* plants, following one year after foliar application of MH (Maleic hydrazide) and MP (manual pruning). * ≡ significant according to Tukey's test at *P < 0.05*.

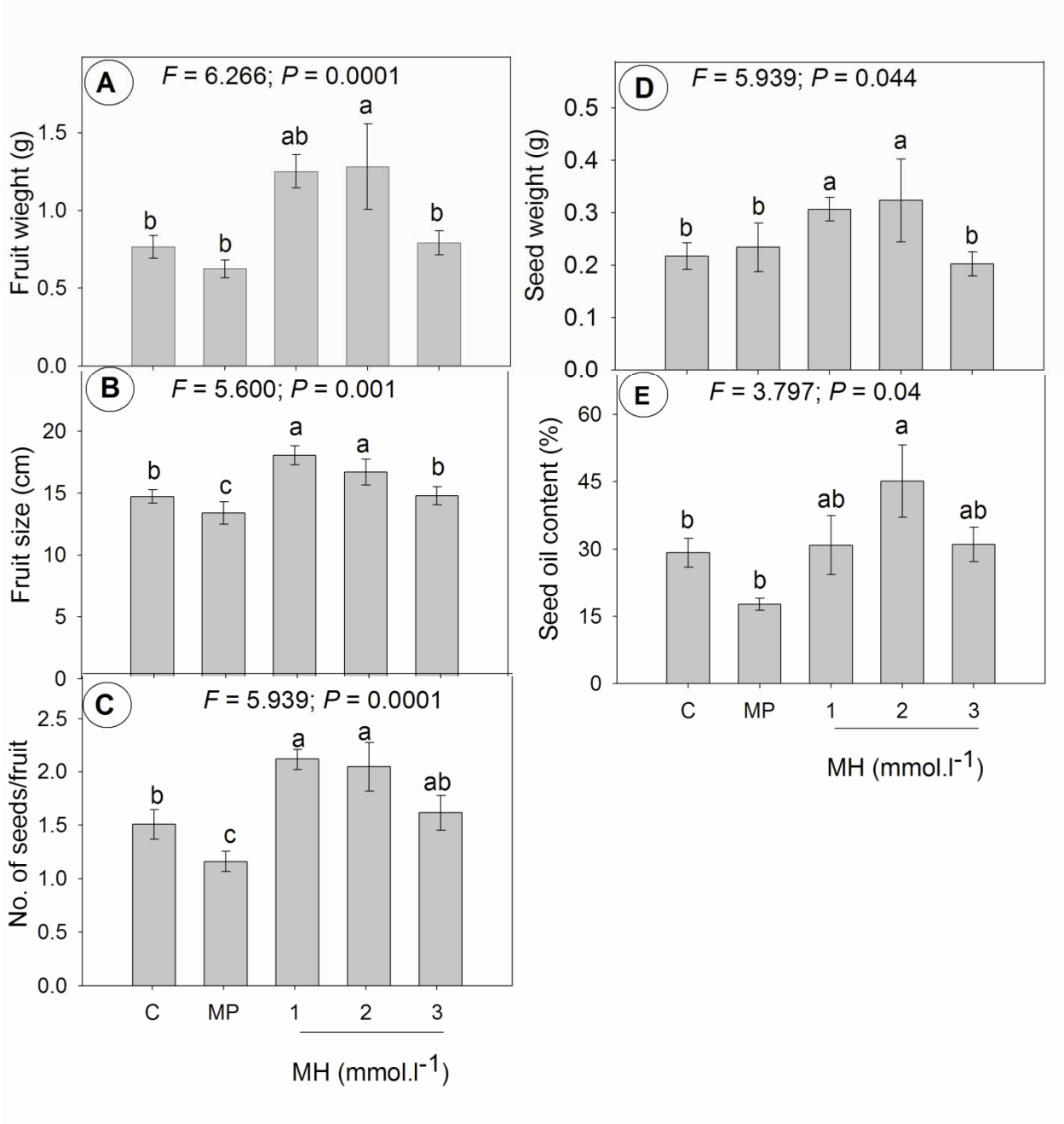

Figure 7.10 Effect of MH (Maleic hydrazide), one year after foliar application and MP (manual pruning), on fruit characteristics of two-year-old plants of *Jatropha curcas*. (A) fruit weight; (B); fruit size; (C) number of seeds per fruit; (D) seed weight; and (E) seed total oil content (%). S.E. bars sharing the same letter(s) are not significantly different from each other according to Tukey's test at *P < 0.05*.

Table 7.5 Effects of PGRs one year after foliar application on free fatty acid content of two-year-old plants of *Jatropha curcas.*

Treatment	Free Fatty Acid Content (%)			
	Palmitic	Linoleic	Oleic	Stearic
Control	18.8	40.0	34.5	6.4
MP	17.3	43.3	32.9	6.4
BA (mmol l^{-1})				
3.0	19.5	37.7	36.1	6.8
6.0	12.3	43.9	33.7	6.9
9.0	18.1	40.1	34.3	6.7
12.0	17.5	43.6	33.1	5.8
TIBA (mmol l^{-1})				
0.5	16.8	48.1	30.0	5.2
1.0	20.8	39.0	32.7	7.5
1.5	17.7	44.2	32.8	5.4
2.0	17.8	43.3	32.9	6.1
DK (mmol l^{-1})				
2.0	21.3	40.4	37.3	6.05
4.0	19.4	39.2	34.8	6.6
6.0	20.1	37.7	35.3	6.9
8.0	19.7	38.6	35.0	6.7
MH (mmol l^{-1})				
1.0	16.8	45.9	32.3	5.0
2.0	19.6	37.0	35.3	8.1
4.0	17.3	44.1	33.1	5.6

Table 7.6 Estimated cost (ZAR/ha) of PGRs application, assuming 6-month-old plants of *Jatropha curcas* with average height of 50 cm, spray rate of 1 l of solution per 50 plants, using compressed air sprayer and plant density of 2500 plant/ha.

PGR	Molecular weight (g)	Spray rate		Cost (ZAR/ha)
		(g/l)	(g/ha)	
BA	225.5	2.7	135.1	11,356.4
TIBA	292.29	1.04	52.5	4,001.50
DK	499.81	2.37	118.5	509.00
MH	112.1	0.45	22.5	105.10

Cost was based on latest pricing of these products by Sigma-Aldrich.

(www.sigma-aldrich.com)

7.4 DISCUSSION

Fruit development is dependent on the interaction of five major classes of plant hormones, each of these include active structures which can have practical applications in fruit production (ZHANG *et al.,* 2008). Crop yields are often increased indirectly by preventing losses, hastening the production cycle, or facilitating mechanical harvest operations. In a few crops, PGRs actually increase plant growth or divert photosynthate to the harvested product so that the actual productivity is increased. There are a variety of reasons to anticipate a significant increase in the commercial use of PGRs and many approaches to discovering and developing these uses (MORGAN, 1980). The effect of PGRs on promotion of branching in *J. curcas* was previously discussed in (**Chapter 4**). The objective of this part of the study was to evaluate the subsequent effect following foliar application of these PGRs and MP on the flowering, fruit set, fruit characteristics and seed total oil content in two-year-old plants of *J. curcas*.

The results showed a significant ($P < 0.05$) increase in the number of flowers per plant and the number of fruit per bunch by foliar application of BA compared to the untreated control and MP (**Table 7.1**). Several studies have shown that application

of exogenous cytokinins increase the number of flower buds in apple (MCLAUGHLIN and GREENE, 1991) and pear trees (ITO *et al.,* 2000). Similar effects have been reported for jojoba *Simmondsia chinensis* (Link) Schneider. by PRAT, *et al*. (2008), who found that seventeen months after application of 100 mg l^{-1} of BA, a significant increase in the number of flowers per branch was observed when compared to the control treatment. He speculated that the significant increase in the number of clusters caused by BA application as being the results of cytokinin action on the axillary meristems, reflected in an enlargement of the axillary meristematic zone. This growth would allow the differentiation of more than one flower per axillary bud, resulting in an increase in total number of flowers produced. Also WERNER *et al*. (2001) found that cytokinins had an important regulatory effect on *Nicotiana tabacum* meristem morphogenesis, enlarging the meristem, which gave a greater probability for the development of flower meristems. TOMPSEET (1977) reported that BA enhanced promotion of flowering in *Picea sitchensis* by a mixture of gibberellins alone or in combination with NAA. In contrast, some studies reported negative effects with synthetic cytokinins as they exhibited inhibitory effects on flowering in apples (SANYL and BENGERTH, 1998) and in *Chenopodium rubrum* (VONDRÁKOVÁ *et al*., 1998). In this study BA produced heavier and bigger fruits when compared to the MP treatment. However, it was not significantly different to the controls (**Figure 7.4A and B**). Also no significant differences were found between treatments with respect to the number of seeds per fruit and seed weight (**Figure 7.4C and D**). These results agree with PRAT *et al*. (2008), who reported no significant differences in the total weight of seeds per plant between the BA treatments and the control in jojoba.

The results demonstrated that foliar application of TIBA produced significantly more flowers per plant and more fruit per bunch compared to the control and MP (**Table 7.2)**. However, there were no variations in fruit set percentage (%) between treatments (**Table 7.2**). Further, TIBA at all concentrations produced significantly heavier fruits compared to the control and MP treatments (**Figure 7.6A**). Several studies reported on the promotive effect of TIBA on flowering and fruiting. A significant increase in flowering in response to TIBA application was reported in sweet cherry *Prunus avium 'Lutovoka'* by GROCHOWSKA *et al*. (2004). In another study with soybean NOODÉN and NOODÉN (1985) found that foliar application of

TIBA increased the number of pods per node. GENG *et al.* (2005) found that, in tulip bulbs, application of TIBA in combination with GA enhanced early flowering and higher flowering rates. Similar results on the effects of TIBA on fruiting was reported in maiden plum *Prunus divaricata*, sour cherry *Prunus avium 'Lutovoka'* and sweet cherry *Prunus avium 'Rivan'* trees (GROCHOWSKA *et al*., 2004). He found that a single foliar application of TIBA increased fruit productivity in all of these species as well as fruit masses in maiden plum *Prunus divaricata*. On average, the increase was about 24% higher than that of the controls. GROCHOWSKA *et al*. (2004) explained his results by the fact that the most characteristic action of TIBA is the inhibition of the polar transport of auxin and, thus, it is categorized as a growth retardant contributing to reduced auxin levels. Therefore, he suggested that the endogenous auxin is a dominant participant in the processes of growth, flowering and fruiting of these three stone-fruit species.

The results showed that no significant differences in fruit size, the number of seeds per fruit and seed weight between TIBA and the control treatments were recorded. However, TIBA at all concentrations produced fruits with significantly ($P < 0.05$) bigger size and with more seeds per fruit compared to the MP treatments (**Figure 7.6B and C**). TIBA was reported to decrease the number of seeds per capsule and seed weight in sesame (DAY, 1999). TIBA at 1.5 and 2 mmol l^{-1} produced physic nut seeds with higher oil content compared to MP (**Figure 7.6E**). No significant differences were found between TIBA at higher concentrations (1.5 and 2 mmol l^{-1}) and the control treatment. However, TIBA at lower concentrations (0.5 and 1 mmol l^{-1}) significantly reduced the seed oil content compared to the control treatment (**Figure 7.6E**).

The results of this study demonstrated that DK at 2 mmol l^{-1} significantly increased the number of flowers per plant and the number of fruit per bunch compared to the control and MP treatments (**Table 7.3**). No significant differences in fruit set percentage between treatments were found (**Table 7.3**). Nevertheless, DK was reported to accelerate floral abscission in citrus (POZO *et al.,* 2004). Foliar application of DK at 2, 4, and 6 mmol l^{-1} produced significantly more seeds per fruit

compared to MP (**Figure 7.8C**). However, there were no significant differences between treatments in fruit weight, fruit size and seed weight (**Figure 7.8A, B and D**). These findings agree with those reported for citrus by POZO *et al.* (2004) that no significant differences in fruit quality were found in response to application of DK. Also RUGINIE and PANELLI (1993) reported for olives that no significant differences were found in fruit weight between DK and the control treatments. However, DK at lower concentration (4 mmol l^{-1}) produced significantly higher seed oil content compared with the control and MP treatments (**Figure 7.8E**). DK at higher concentration (8 mmol l^{-1}), however, reduced the seed oil content significantly compared to the control (**Figure 7.8E**). In contrast, for olives (*Olea europaea* L.) RUGINIE and PANELLI (1993) reported no significant differences in oil content between DK and control treatments.

The results demonstrated that the number of flowers per plant was significantly increased by 1 mmol l^{-1} MH compared to the control treatment and MP (**Table 7.4**). These results agree with those of ITO *et al.* (2000) who found that in Japanese pear foliar application of MH increased the number of laterally-borne flower buds on the shoots. They suggested that MH may increase cytokinin levels in lateral buds and thus as a result increase the number of flower buds. In this study MH at 2 mmol l^{-1} produced significantly ($P < 0.001$) heavier fruit compared to the control, MP and MH at higher concentration (4 mmol l^{-1}) (**Figure 7.10A**). Foliar application of MH at 1 and 2 mmol l^{-1} produced fruits significantly bigger, more seeds with heavier seed weight compared to the control, MP and MH at higher concentration (4 mmol l^{-1}) (**Figure 7.10B, C and D**). MH at 2 mmol l^{-1} produced seeds with a significantly higher oil content (**Figure 7.10E**).

The results showed that only four Free Fatty Acids (FFA) were found in the study sample and the dominant FFA was linoleic acid followed by oleic acid, palmitic acid and stearic acid (**Table 7.5**). There was no variation detected in the FFA content between treatments (**Table 7.5**). These results do not agree with those of ADEBOWALE and ADEDIRE (2006) who, in addition to the four FFA detected around 4.7% of arachidic acid with a dominant component of stearic acid, which

ranked the lowest in our study sample. These discrepancies could be due to differences among cultivars of *J. curcas* or to the methodology used to extract and analyse fatty acids.

Quantitative data on yield increases resulting from growth regulator applications are most commonly available at the time a substance is being cleared for use (proof of efficacy). Well established products are maintained in use by grower experience rather than by newly published data. In the hands of producers, growth regulators must prove themselves as the bottom line of a financial balance sheet (MORGAN, 1980). In this respect, **Figures 7.3, 7.5, 7.7 and 7.9** compare seed weight and seed oil content which are the major economic yield components for *J. curcas*; **Table 7.6** compares the application cost (ZAR/ha) of the PGRs used in this study. The results revealed that MH was the only PGR that gave significant increase in yield component and simultaneously was the least expensive PGR. Therefore, the results from this **Chapter** in combination with the results from **Chapter 4** suggests further thorough investigation into MH interactions in this plant in order to be registered as an efficient chemical pruning agent, yield promoter and cost effective PGR for *J. curcas* seed production improvement.

8 Promotion of seed germination and seedling growth of *Jatropha curcas*

8.1 INTRODUCTION

There are many agronomic and biological constraints limiting *Jatropha curcas* L. seed yield, making the crop not ready for commercial oil production. Some of the inherent problems associated with *J. curcas* seeds are low germination ability and loss of seed viability if not carefully stored. The seed deteriorates with storage resulting in poor germination and weak seedling growth (SWARUP, 2006; PARAMATHMA and SRIMATHI, 2006; SWAMY and SINGH, 2006). Effective propagation of healthy plants is a prerequisite for the introduction of a new plant into a market, for development by breeders, and propagation for production by growers. High seedling vigour is a prerequisite for plant establishment (VAN STADEN *et al*., 2006). Numerous studies show that application of bio-stimulants and PGRs may increase the germination ability of seeds and play an important role in seedling establishment (RUSSO and BERLYN, 1990; CRUNKILTON *et al*., 1994; SWAMINATHAAN and SRINIVASAN 1996; VAN STADEN *et al*., 2006). Moreover, smoke technology has the potential to be used in the horticultural and agricultural industry for the production of healthier and more vigorous crops (LIGHT and VAN STADEN, 2004).

In this study, the viability, moisture content and imbibition of *J. curcas* seeds were investigated. The effect of smoke (aerosol smoke and smoke water), IBA, NAA and KNO_3 on seed germination and seedling growth of *J. curcas* were tested.

8.2 MATERIALS AND METHODS

8.2.1 Seed source

Fruits were collected in May 2007 from a two-year-old monoculture crop of *J. curcas* established from seeds at the University of KwaZulu-Natal Research Station Ukulinga, Pietermaritzburg, South Africa (30˚41' E, 29˚67' S; 781 m a.s.l). Seeds were stored under laboratory conditions at 20 ℃ and kept in brown paper bags until used in these experiments in November 2007 (6-month-storage).

8.2.2 Viability test

Seed viability was determined from three replicates of 30 seeds each using TTC solution. The seeds were shelled and imbibed for 24 h in water. After cutting longitudinally, to expose the embryo, they were soaked in 1% solution of TTC for 24 h at 25 ± 0.5 ℃ in the dark. Seeds with red-stained embryos were recorded as being viable (ISTA, 1999). Viability percentage was calculated as the number of red-stained embryos to the total number of embryos.

8.2.3 Moisture content

The moisture content of the seeds was determined by drying seeds (three replicates of 30 seeds each) at 110 ˚C for 48 h to constant weight. The moisture content was expressed as a percentage of fresh weight (KULKARNI *et al*., 2007).

8.2.4 Imbibition

Four replicates of 15 seeds each were placed in 9 cm Petri dishes on two layers of filter paper (Whatman No. 1) moistened with 15 ml distilled water and allowed to imbibe at room temperature (25 ˚C). The increase in seed weight was determined after 2, 4, 8, 12, 24, 36, 48, 72 and 96 h. Seeds were blotted dry before weighing and

thereafter returned to the wet filter paper. The amount of water imbibed by the seeds was graphically represented as the percentage increase over the initial seed weight.

8.2.5 Germination tests

8.2.5.1 Aerosol smoke

Intact and shelled seeds with four replicates of 15 seeds each were placed separately in sieves and exposed to cool aerosol smoke for 0, 30, 60 and 90 min (SPARG *et al*., 2006). This was achieved by placing the sieves inside a chimney, 150 cm above slow-burning smouldering semi-dry and dry grass, leaves and branches collected from the University of KwaZulu-Natal Botanical Garden. After exposure to smoke, seeds were washed with distilled water and placed on a roll of paper towel moistened with distilled water. These rolls were placed inside plastic bags and incubated at 25 C° under 16-h photoperiod provided by Osram® 75 W cool white fluorescence tubes providing an irradiance of 16 µmol m^{-2} s^{-1} at shelf level.

8.2.5.2 Smoke solution, nitrogen salts and plant growth regulators

The smoke solutions used were obtained from the dilution of an aqueous smoke extract produced from burnt *Themeda triandra* material as outlined by BAXTER *et al*. (1994). KNO_3, NAA and IBA solutions were prepared in the laboratory. Seeds were decontaminated by immersing them in 0.1% mercuric chloride for 2 min and then rinsing with distilled water. Three replicates of 30 seeds each were soaked for 24 h in either: (1) distilled water using intact-seeds representing, Control-1 treatment and referred to hereafter, as C1, (2) distilled water using shelled-seeds, representing Control-2 treatment and referred to hereafter, as C2, (3) SW (1:500, 1:1000 and 1:1500), (4) KNO_3 (10^{-5}, 10^{-6} and 10^{-7} M), (5) IBA (10^{-5}, 10^{-6} and 10^{-7} M), and (6) NAA (10^{-5} and 10^{-6} M). The treated seeds were then placed on paper towels, wetted with distilled water inside plastic bags and kept moist for five days. Germination was recorded daily and was considered complete once the radicle had protruded about 2 mm in length. The experiment was continued for five days when all treatments reached full germination. However, for the aerosol smoke treatments, observation continued for 21 days. Germination percentage was calculated as the number of

seeds germinated to the total number of seeds placed for germination. The germination data were arcsine transformed for statistical analysis (DEZFULI *et al*., 2008). Mean germination time (MTG) was calculated using the equation: MGT = Σ *nxd*/*N* where, where *n* is the number of seeds germinated between observation intervals, *d* the incubation period in days after time of observation and *N* the total number of seeds in the sample that germinated in the treatment (BALESTRI and BERTINI, 2003).

8.2.6 Seedling growth

After germination the seedlings were planted in plastic trays (**Figure 8.1**) (20x15x5 cm^3) in a shade house in the Botanical Garden of the University of KwaZulu-Natal, Pietermaritzburg, with an average light photosynthetic photon flow density of 331 µmol m^{-2} s^{-1} at mid-day. The soil mixture in each plastic tray was compost: bark (chipped and decomposed pine): LAN (limestone ammonium nitrate): [2:3:2 NPK (nitrogen, phosphorus, potassium)] (4:1:0.1:0.1). Each tray represented a replicate containing ten seedlings, and were placed randomly and watered twice-a-week with tap water.

Figure 8.1 Seedlings of *Jatropha curcas* grown in trays in a shade house.

After three months, seedling growth parameters such as mass, the number of leaves, stem width, stem length, root length, leaf area (LA) and vigour index (VI) were measured. Vigour index (VI) was calculated as: percentage germination x (stem length + root length) (DHINDWAL *et al*., 1991). However, the number of roots was not considered for the growth trait measurements because the plant has a constant number of roots (one taproot with four lateral roots) (HELLER, 1996).

8.2.7 Data analysis

Data were subjected to one-way analysis of variance (ANOVA) using SPSS® (SPSS nc., Chicago, USA) release 15 statistical software. The *post hoc* Tukey's test was carried out and a significance level of $P < 0.05$ was used for all statistical tests.

8.3 RESULTS

8.3.1 Seed viability, moisture content and imbibition

The mean viability and moisture content percentages of the fresh seeds were 90% and 11.72%, respectively. The imbibition curve for the shelled-seeds was steep at the beginning, however, within 24 h, the net water uptake reached a plateau and germination occurred within 48 h (**Figure 8.2**). The imbibition curve for the intact-seeds remained steep up to 96 h and germination occurred at 105 h (**Figure 8.2**).

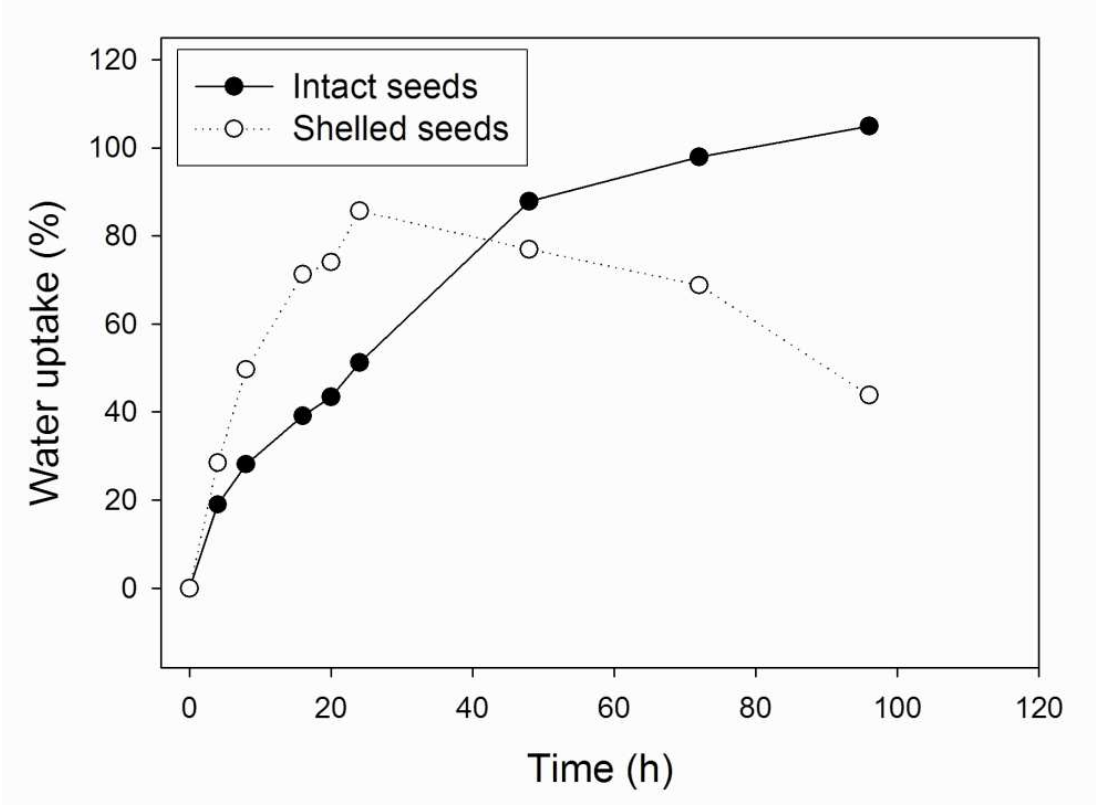

Figure 8.2 Imbibition curve for *Jatropha curcas* seeds at 25 ℃.

8.3.2 Germination

Seeds exposed to AS (aerosol smoke) failed to germinate over the whole period of the study (three months). There were no significant differences in germination between the control treatments (C1, C2) and the other treatments where the seeds were soaked for 24 h in either distilled water, SW, KNO_3, IBA, or NAA (**Table 8.1**). However, the control treatment C2 (shelled-seeds) had a lower MGT compared to the control treatment C1 (intact-seeds) and the other treatments (**Table 8.1**).

Table 8.1 Effects of SW (smoke water), KNO_3, IBA, and NAA on seed germination of *Jatropha curcas*. All germination occurred within nine days.

Treatment	Germination (%)	MGT (days)
C1 (control of intact-seeds)	93.7 ± 0.48	5
C2 (control of shelled-seeds)	95.0 ± 0.48	2
SW 1:500	95 ± 0.57	3
SW 1:1000	98.3 ± 0.25	3
SW 1:1500	100 ± 00	3
KNO_3 10^{-5} M	98.3 ± 0.25	3
KNO_3 10^{-6} M	100 ± 00	3
KNO_3 10^{-7} M	100 ± 00	3
IBA 10^{-5} M	96.7 ± 0.5	3
IBA 10^{-6} M	98.3 ± 0.25	3
IBA 10^{-7} M	98.3 ± 0.25	3
NAA 10^{-5} M	96.7 ± 0.5	3
NAA 10^{-6} M	100 ± 00	3

Means ± S.E values showed no significant differences between the treatments.

8.3.3 Seedling growth and vigour

8.3.3.1 Smoke water (SW)

Smoke water at a dilution of 1:500 produced significantly heavier seedlings with more leaves, wider and longer stems, longer roots, and a higher vigour index (VI) compared to the control treatments (C1 and C2) (**Figure 8.3A and Figure 8.4A-E)**. However, other than leaf area, there were no significant differences between the three dilutions of SW for all parameters measured (**Figure 8.4A-G**).

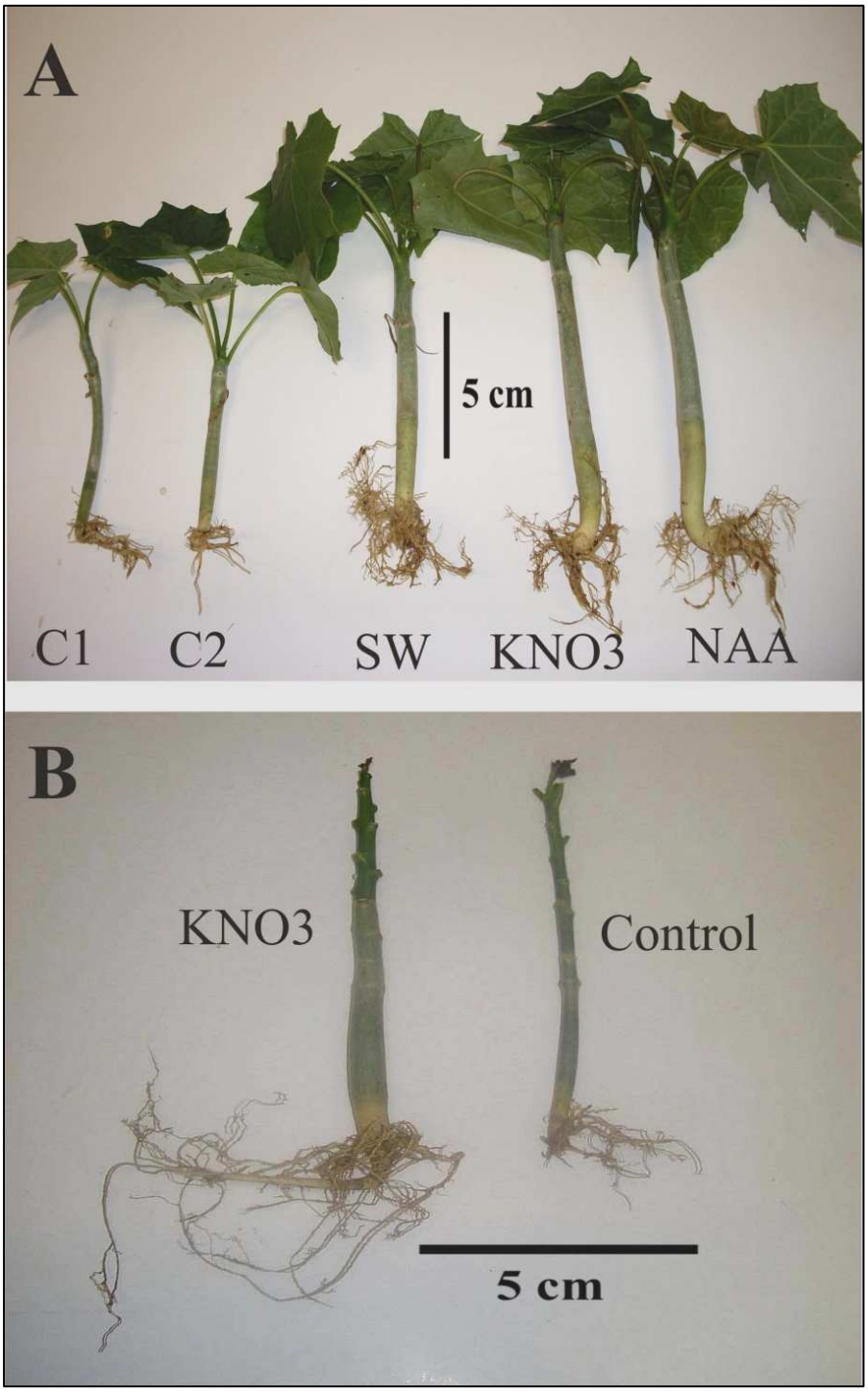

Figure 8.3 Three-month-old *Jatropha curcas* seedlings as influenced by 24 h pre-sowing seed-soaking treatments of SW (smoke water), KNO_3 and NAA. (A) C1 (control of intact-seeds), C2 (control of shelled-seeds), SW, KNO_3 and NAA; (B) Root length of control of intact-seeds (C1) and KNO_3 treatments.

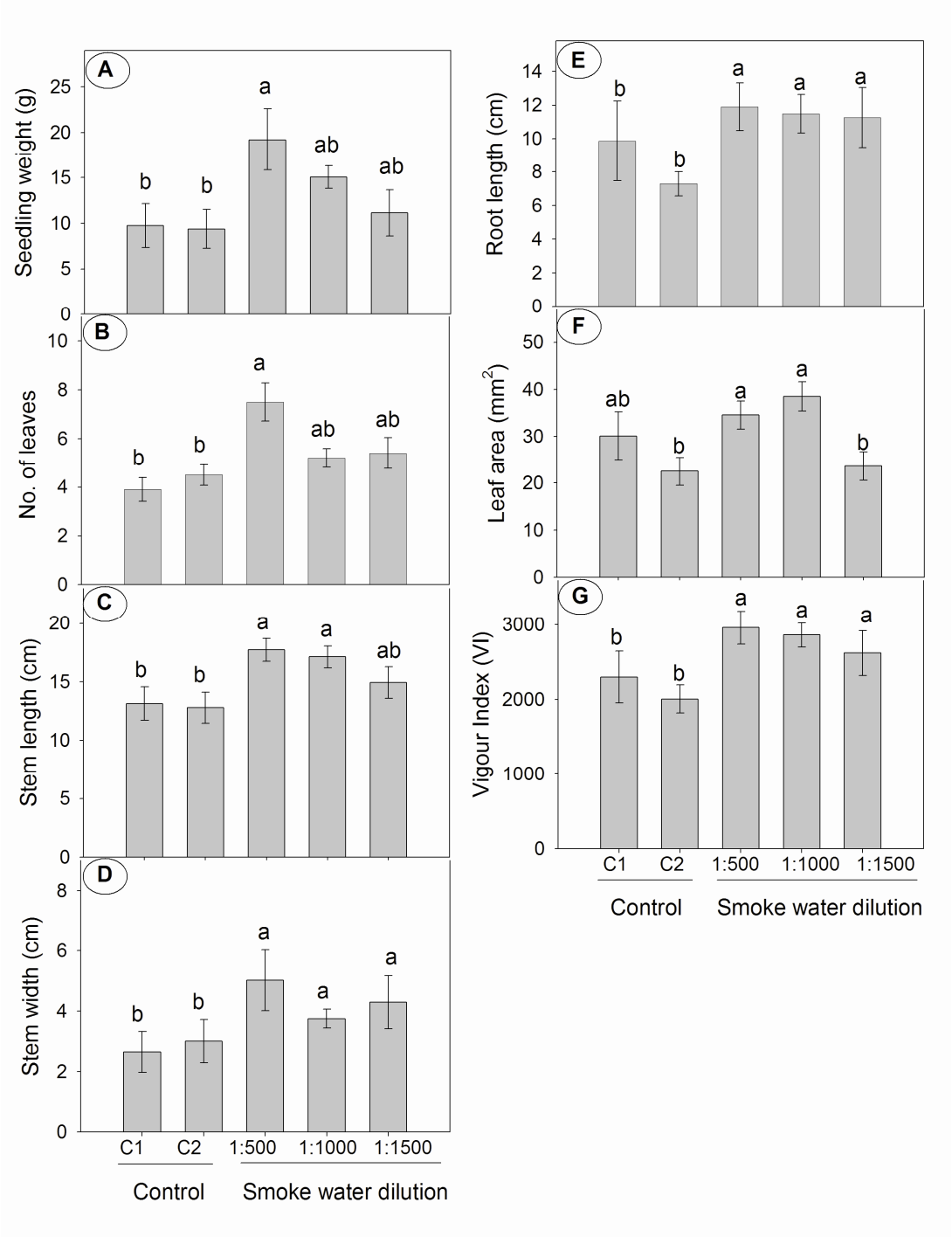

Figure 8.4 Influence of SW (smoke water) (1:500, 1:1000, and 1:1500 V/V) on seedling growth of *Jatropha curcas* under shade house conditions. Values are means ± standard error. Bars with different letter(s) are significantly different to each other according to Tukey's test ($P < 0.05$).

8.3.3.2 Potassium nitrate

Potassium nitrate at a concentration of 10^{-5} M produced significantly heavier seedlings with wider stems, longer roots and a higher vigour index compared to untreated control treatments of intact- and shelled-seeds, respectively (**Figure 8.5A, D, E and G**). However, with the exception of leaf area, there were no significant differences between the three concentrations of KNO_3 used (**Figure 8.5A-G**).

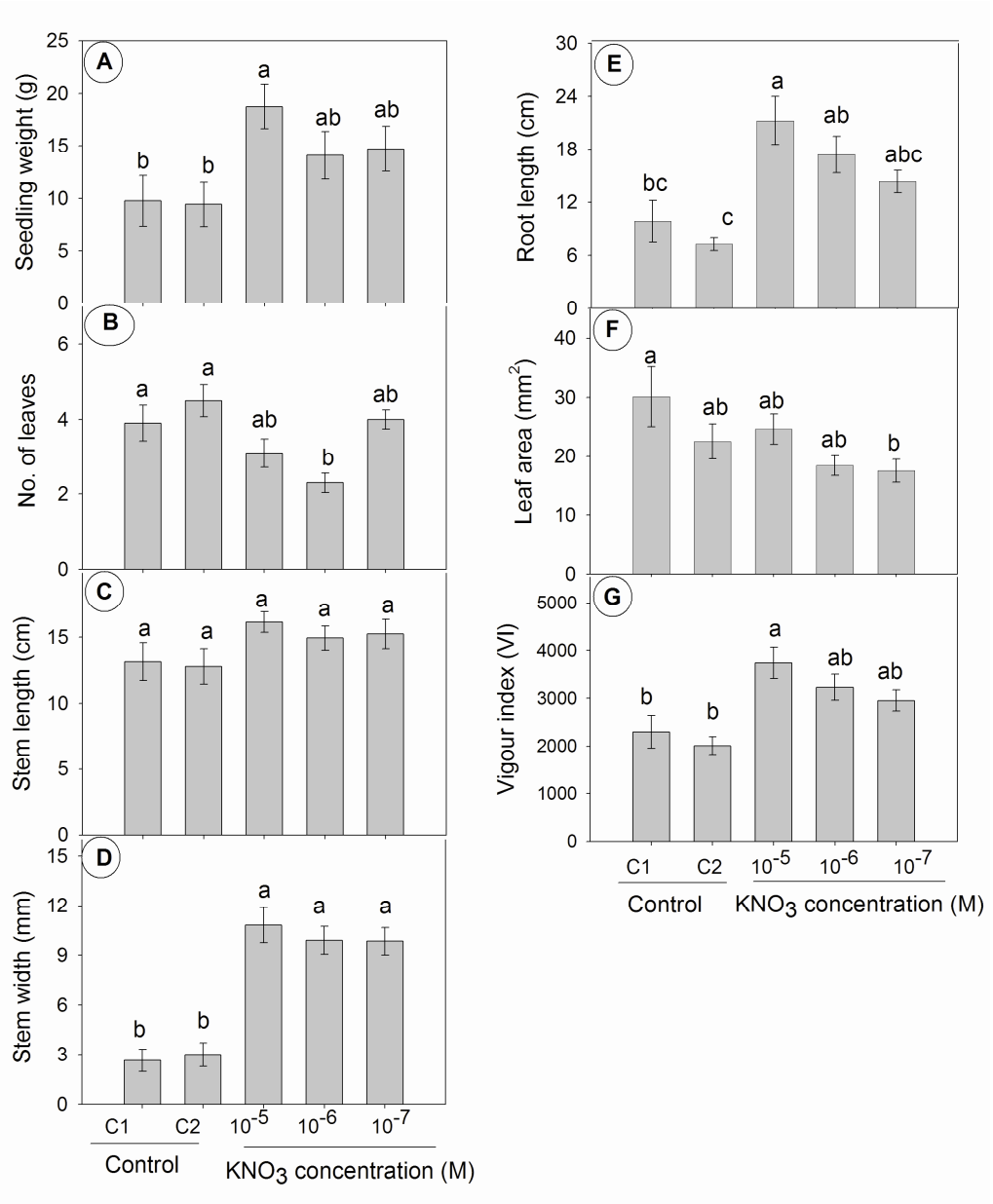

Figure 8.5 Influence of KNO_3 (10^{-5}, 10^{-6}, and 10^{-7} M) on seedling growth of *Jatropha curcas* under shade house conditions. Values are means ± standard error. Bars with different letter(s) are significantly different to each other according to Tukey's test ($P < 0.05$).

8.3.3.3 Indole-3-butyric acid

Indole-3-butyric acid at all concentrations used produced significantly thicker stems than the untreated control treatments of intact- and shelled-seeds, respectively (**Figure 8.6D**). At 10^{-7} M, significantly longer roots were produced than in the control of untreated shelled-seeds (**Figure 8.6E**).

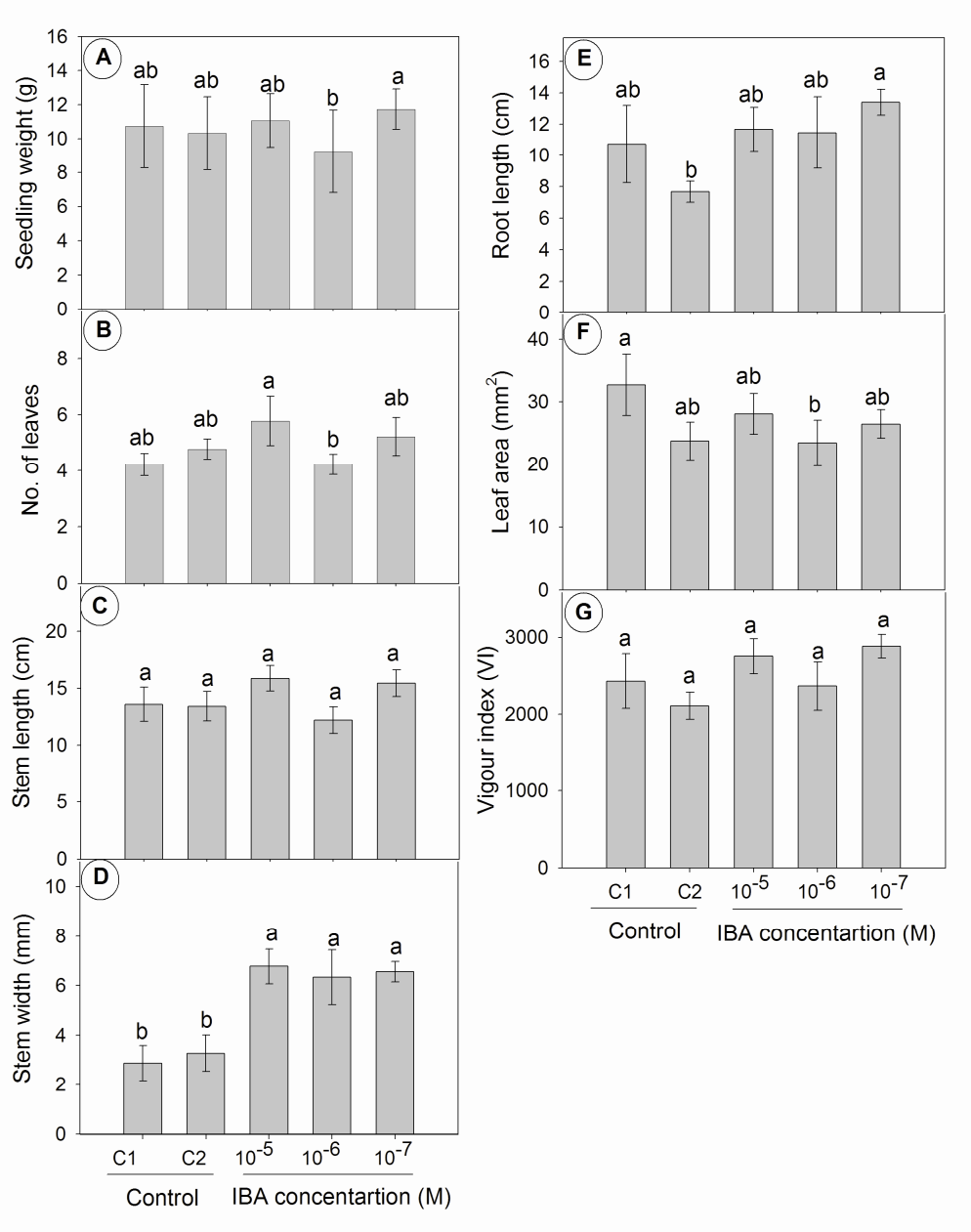

Figure 8.6 Influence of IBA (10^{-5}, 10^{-6}, and 10^{-7} M) on seedling growth traits of *Jatropha curcas* under shade house conditions. Values are means ± standard error. Bars with different letter(s) are significantly different to each other according to Tukey's test ($P < 0.05$).

8.3.3.4 Naphthalene acetic acid

Naphthalene acetic acid at 10^{-5} and 10^{-6} M produced significantly heavier seedlings with longer stems, wider stems, longer roots and had a higher vigour index compared to untreated control treatments of intact- and shelled-seeds, respectively (**Figure 8.7 A, B, D, E and G**).

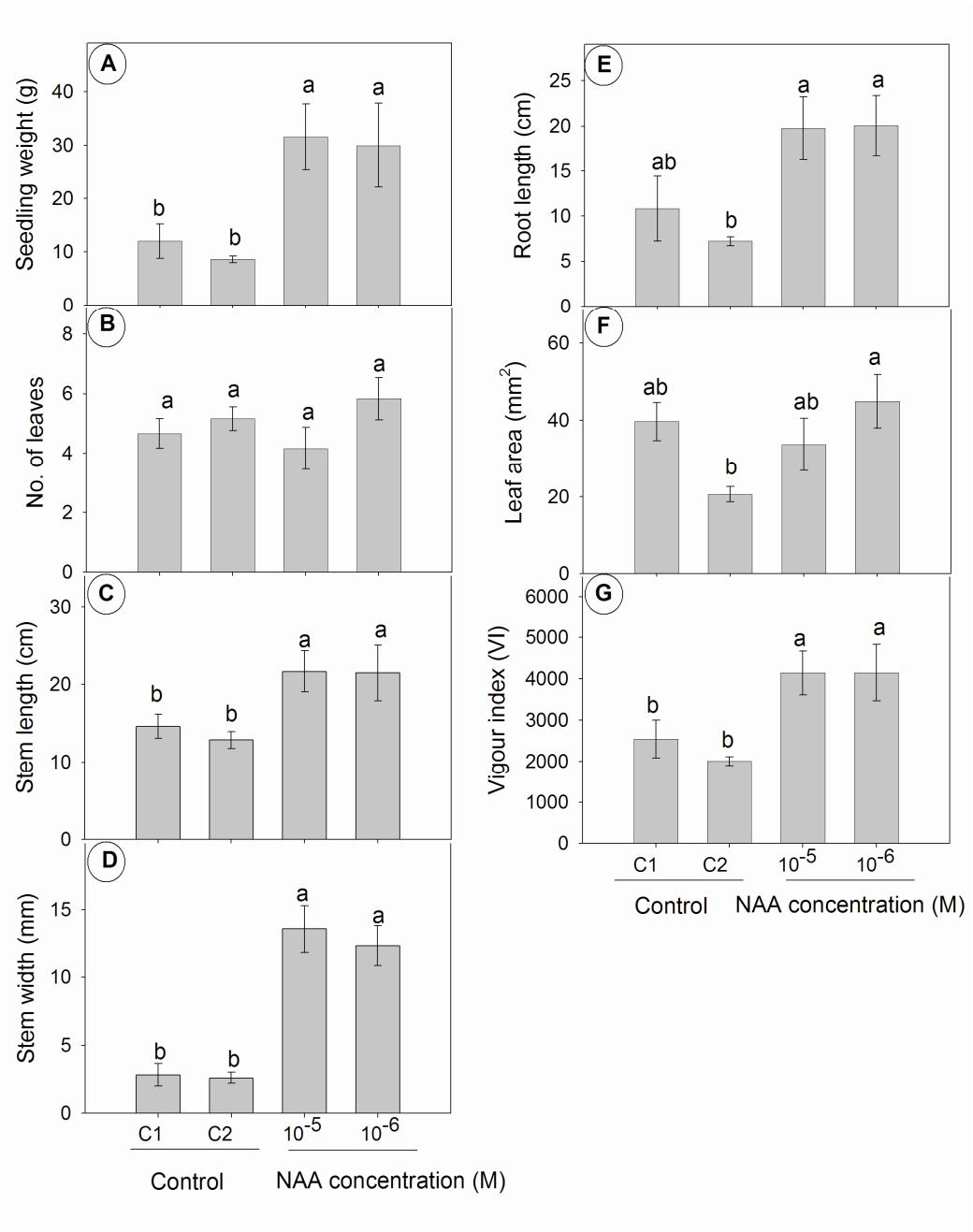

Figure 8.7 Influence of NAA (10^{-5} and 10^{-6} M) on seedling growth traits of *Jatropha curcas* under shade house conditions. Values are means ± standard error. Bars with different letter(s) are significantly different to each other according to Tukey's test (P < 0.05).

8.4 DISCUSSION

The first process that occurs in germination is water uptake, involving mainly imbibition. As the water content rises, the imbibitional force rapidly decreases, so that the rate of water uptake slows down, and osmotic forces become relatively more important and determine the final water content reached in the hydration phase. During this phase, the seed coat is frequently the limiting factor and in such cases removal or puncturing of the seed coat significantly speed up the rate of water uptake (STREET and ÖPIK, 1970). In agreement, the results from this study show that the removal of the seed coat accelerated the imbibition and shortened the germination time (2 days) for the shelled-seeds compared to the water soaked intact-seeds (5 days) (**Table 8.1**).

Seeds exposed to aerosol smoke failed to germinate over the whole study period (three months). In this case the negative results can be due to some physical conditions from the smoking treatment. However, the contrasting effects of smoke on germination and its inhibitory impact were suggested to be due to presence of ranges of germination inhibitors for which there are species-specific responses (DREWES *et al*., 1995). Also DAWS *et al*. (2007) reported that while smoke stimulated germination in a number of species it also had negative impacts on other species.

The results show that SW, KNO_3, NAA and IBA had no influence on seed germination of *J. curcas*. However, they influenced the subsequent developmental stages of the seedlings. SW at a dilution of 1:500 produced heavier seedlings with more numerous leaves, wider stems, longer stems and roots, and had a higher vigour index (VI) compared to the control treatments (**Figures 8.3; 8.4A-E ;8.4G**). These results are in line with VAN STADEN *et al*., (2006) who reported that smoke water at a dilution of 1:500 significantly increased seedling mass and vigour index of okra (*Abelmoschus esculentus* L. Moench), tomato (*Solanum lycopersicum* L.) and maize (*Zea mays* L.) compared to the untreated controls. Similar results had been reported (KULKARNI *et al*., 2006 and KULKARNI *et al*., 2007) in rice (*Oryza sativa* L.) and Dioscorea (*Dioscorea dregeana* Kunth Dur. and Schinz). Furthermore, in a study by TAYLOR and VAN STADEN (1996), they had shown that a smoke extract

stimulated root formation in mung bean (*Vigna radiata* (L.) Wilczek.), indicating that smoke constituents may play a significant role in promoting rooting.

The results show that KNO_3 at a concentration of 10^{-5} M produced seedlings significantly heavier with wider stems, longer roots and a higher vigour index (**Figure 8.5A, D, E and G)**. This is in line with KATTIMANI *et al*. (1999) who found that in ashwagandha (*Withania somnifera* Daunal.) seeds soaked in 1% KNO_3 solution for 24 h produced more vigorous seedlings, had higher dry matter accumulation and longer root length compared to water soaked seeds. Similar results have been reported in *Angelica gluaca* by BUTOLA and BADOLA (2004) who reported that plant height and root length were positively elongated by KNO_3.

The study showed that the three IBA concentrations used produced significantly thicker stems than the control treatments (**Figure 8.6D**). NAA at 10^{-5} and 10^{-6} M resulted in significantly heavier seedlings with longer stems, wider stems, longer roots and a higher vigour index compared to the control treatments (**Figure 8.7A, B, D, E and G**). These results are in line with BALESTRI and BERTINI, (2003) who found that in *Posidonia oceanica* treatments of the seed with NAA and IBA initiated roots faster than untreated controls. Further, they reported that after five months, the roots of seedlings exposed to NAA and IBA were significantly longer compared to the controls. Likewise, REED *et al*., (1988) found that a single application of a commercially-available product containing both NAA and IBA increased root development in the surfgrass *Phyllospadix torrey* S. Watson.

9 General conclusions and recommendations

9.1 RESEARCH HIGHLIGHTS

The overall aim of this PhD study was to develop measures to expedite research and development on improving seed production of *J. curcas* L. to become commercially viable. The two strategies identified to achieve this aim were manipulation of pollination and regulation of vegetative growth.

9.2 GENERAL CONCLUSIONS

The general conclusions derived from this study are:

- Seed coat removal can accelerate imbibition thereby lead to faster germination of *J. curcas* seeds;
- SW, NAA and KNO_3 proved to be very effective in improving seedling growth and vigour in this plant;
- *Jatropha curca*s is self-compatible and reproduces through a mixture of self- and outcross-pollination;
- Pollination by insects was essential for producing a large quantity of seed of good quality;
- Honeybees were effective pollinators;
- Fruit arising from self-pollination were almost as numerous and as large as those arising from cross-pollination;
- The absence of detectable inbreeding depression for most measured traits suggests that open-pollinated seed (which would include some self-fertilised seed) is probably of high enough quality for planting orchards of *J. curcas*;

- Pollen viability, *in vitro* and *in vivo* pollen germination and pollen tube growth of *J. curcas* were detailed in this study;
- TTC salt is a reliable stain for pollen viability tests in this plant;
- Pollen from hermaphrodite flowers is less viable and has poor *in vitro* germination and is thus not reliable for fertilization;
- Boric acid, calcium nitrate and sucrose are essential requirements for *in vitro* pollen germination;
- Exogenous hormones IAA can play an important role for *in vitro* studies;
- Winter- and summer- pruning can improve branching compared to un-pruned plants. However improvement on fruit bearing is expected only on the subsequent seasons; PGRs tested in this study were effective in promoting branching of *J. curcas*. However, their influence was much more pronounced in the field, achieving significantly greater numbers of branches than manual pruning and untreated plants;
- Therefore, PGRs can become a valuable tool for promoting *J. curcas* branching under field conditions;
- DK and MH can be applied to achieve good branching and higher oil content at lower cost compared with BA and TIBA;
- The four PGRs used in this study have no influence on the Free Fatty Acid content of *J. curcas* oil; and
- There was strong suggestion for further thorough investigation into MH interactions in this plant as it may provide an efficient chemical pruning agent, yield promoter and cost effective PGR for *J. curcas* seed production improvement.

9.3 RECOMMENDATIONS

- For rapid germination, seed coat removal is recommended;
- For obtaining vigorous seedlings and consequently, good crop establishment and yield, pre-sowing treatments of seeds with SW, NAA or KNO_3 is recommended;
- For obtaining higher fruit set and good quality seed, it is recommended to provide beehives for large mono-culture plantations;
- For orchard management for fruit yield in *J. curcas* promotion of cross-pollination does not have to be a priority; and
- For obtaining good branching, better seed quality and quantity and more oil, foliar application of DK or MH is recommended.

9.4 FUTURE PROSPECTS

- Much work still remains to be done to maximize the overall productivity of *J. curcas*. The bulk of this work is probably best done on the area of genetic improvement taking advantages of plant biotechnology. Specifically, future efforts should focus on modifying *J. curcas* genes to increase the number of fruit/plant;
- Meanwhile, however, it is important to continue a certain amount of work in the area of growth regulation and pollination to resolve many questions raised during this PhD study. Specifically some work to overcome the male sterility in the hermaphrodite flower and to improve the male:female flower ratio;
- It is beneficial to undertake studies to determine the actual contribution of beehive supplementation to an orchard. This was not possible to cover in this study due to the limited area of the study site;
- This study covered the breeding system through bagging experiments followed by confirmation through an *in vivo* investigation. This shows that

seeds are potentially produced through pollinator-mediation. However, it would be good to confirm that the plant has a mixed mating system and to determine the exact outcrossing rate using molecular markers;

- It may be worthwhile to try the other PGRs that were not used in this study such as GA, paclobutrazol, meta-topolin, zeatin or any other potential PGRs;

- Since the plant hormones act antagonistically it might be useful to combine two or three PGRs together to achieve a balanced growth that would satisfy the commercial properties. For example in this study some of the PGRs caused a dramatically horizontal branch growth which might negatively affect the performance of many cultural practices;

- A lot of improvements need to be made on the flowering and fruit set of this plant. Some of this improvements should be in the area of shortening the harvest season to reduce the cost of the harvest;

- Classical breeding and selection methods could be implemented for improvement of yield. This would entail initiating a collection of diverse *J. curcas* germplasm, screening of these plants for high yielding genotypes, and selecting those with favourable characteristics such as seed and oil yield;

- Further, it has been reported that hybridization between *J. curcas* and *J. intigerrima* resulted in F2 progeny that produced mostly bisexual flowers. This would be highly desirable, but these progeny would have to be assessed for growth characteristics and oil content to ascertain if they would be suitable;

- In several monoecious plants, perfect hermaphrodite flowers are initiated at the time of floral morphogenesis, but one sex organ fails to develop. As in *J. curcas*, this results in separate male and female flowers on the same plant. Application of IAA (or ethylene) at the bisexual stage may result in female flowers being produced; However, it would also be necessary to ensure that sufficient male flowers exist in the population to ensure fertilization of female flowers.

- Moreover, it may be possible to manipulate the identity of flowers by altering the expression patterns of specific genes. This could potentially lead to many more hermaphrodite and/or pistilate flowers being produced.

10 References

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