Pollen viability, pollen germination and pollen tube growth in the biofuel seed crop *Jatropha curcas* (Euphorbiaceae)

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Abstract

The fate of pollen and pollen tubes can have a profound effect on fruit and seed production. Experiments were conducted to investigate pollen viability, *in vitro* pollen germination and *in vivo* pollen tube growth in the biofuel seed crop *Jatropha curcas*. It was possible to 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 and indole-3-acetic acid (IAA) treatments. Pollen from hermaphrodite flowers had 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 h after pollination, thus confirming earlier reports of self-compatibility in this species.
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Keywords: IAA; *In vitro* pollen germination; *In vivo* pollen tube growth; Pollen pistil interaction; Pollen viability; TTC

1. Introduction

Jatropha curcas L. (Euphorbiaceae) is a crop with potential for biodiesel production in arid and semi-arid regions (Achten et al., 2008; Augustus et al., 2002; Azam et al., 2005; Heller, 1996). The co-products from *Jatropha* oil extraction are suitable for cellulosic ethanol production, thus meeting the alcohol demands of the biodiesel transesterification process (Visser et al., 2011). Plants are monoecious, but hermaphrodite flowers are occasionally produced (Abdelgadir et al., 2009; Dehgan and Webster, 1979). The species is self-compatible but requires pollinator visits to mediate pollen transfer among the unisexual flowers for seed production (Abdelgadir et al., 2009). Self-pollination leads to marginally lower fruit set than does cross-pollination (Abdelgadir et al., 2009). There is currently very limited information about pollen quality and its measurement, and pollen tube growth rates following self- and cross-pollination.

Effective pollination is a prerequisite for fruit- and seed-set in most plants, and information on pollen biology, including pollen viability and pollen tube growth, is required for any rational approach to increase productivity (Bolat and Pirlak, 1999; Cruzan, 1989; Shivanna, 2003). Pollen viability can be evaluated by: (1) staining techniques; (2) *in vitro* and *in vivo* germination tests; or (3) analyzing final seed set. The choice of method depends on the crop or species (Dafni and Firmage, 2000; Dafni et al., 2005). 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. In many species, *in vitro* pollen germination is dependent on the addition of key substrates such as calcium nitrate to the germination media (Steer and Steer, 1989). Indole-3-acetic acid (IAA) plays an essential role in plant sexual reproduction by controlling the development of stamens and ovaries; promoting the maturation of egg cells; and inducing the axial polarity and polar development of the embryo (Wu et al., 2008a). It has also been suggested that IAA promotes pollen tube growth in the pistil (Aloni et al., 2006; Kovaleva and Zakharova, 2003). Wu et al. (2008b) reported that IAA stimulated pollen tubes

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to grow long and straight. Pollen tube growth can affect the outcome of self- versus cross-pollination, as pollen tubes from self-pollen may grow slower or have higher rates of attrition than those from cross pollen (Aizen et al., 1990).

In this study, pollen viability, pollen germination and pollen tube growth following cross- and self-pollination were investigated in *J. curcas*. Four basic questions were addressed: (1) What is the level of viability of pollen from male and hermaphrodite flowers?; (2) Which staining technique is reliable for testing *J. curcas* pollen viability?; (3) What is the optimum medium for *in vitro* pollen germination and pollen tube growth?; and (4) Is there any evidence that IAA plays a role in enhancing pollen germination and pollen tube growth in this plant?

2. Materials and methods

2.1. Study species

Jatropha curcas is a monoecious shrub or small tree producing racemes of mostly unisexual flowers (Fig. 1A). Female and male flowers (Fig. 1B and C, respectively) are produced in the same inflorescence. Hermaphrodite flowers (Fig. 1D) are occasionally present (Abdelgadir et al., 2009; Dehgan and Webster, 1979). The average male to female flower ratio is 16:1 (Abdelgadir et al., 2009). Male flowers are salver-shaped, with ten stamens arranged in two whorls of five each. The pollen grains (Fig. 1F) are inaperturate and range in number between 120 and 160 per anther. The diameter of pollen grains is ca. 81–95 µm (Bhattacacharya et al., 2005; Kaur et al., 2011; Raju and Ezradanam, 2002). Female flowers (Fig. 1G) are larger with an ovary (Fig. 1G and H) consisting of three carpels, each with a single locule containing one ovule (Fig. 1I) (Chang-Wei et al., 2007; Raju and Ezradanam, 2002). The species has been shown to be effectively pollinated by diurnal insects (Abdelgadir et al., 2009) but is potentially also pollinated by nocturnal visitors since the flowers remain open during the night. However, nectar production in male and female flowers peaks in the morning, declines in the afternoon and reaches the lowest level at night over the whole anthesis period (Luo et al., 2011).
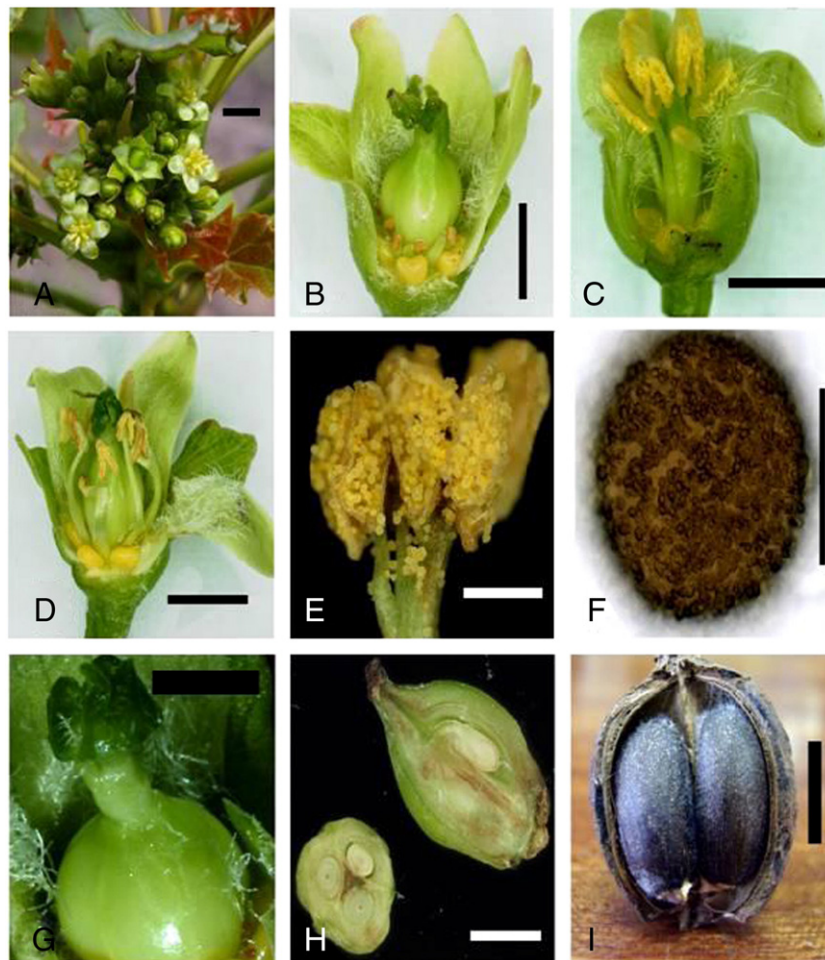

Fig. 1. Pollen, floral organs, flowers, inflorescence, fruit and seeds of *Jatropha curcas*, (A) racemose inflorescence; (B) female flower; (C) male flower; (D) hermaphrodite flower; (E) anthers (F) pollen; G) stigma and ovary; (H) longitudinal and cross sections of ovary five days after pollination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

2.2. Study site and plant materials

Branches with several inflorescences were collected from 50 to 60 trees in a monoculture plantation at the University of KwaZulu-Natal Agricultural Research Station (Ukulinga), (30° 41′ E, 29° 67′ S and 781 m), Pietermaritzburg, South Africa. The plantation was established from seeds obtained from Owen Sithole College of Agriculture, South Africa. The original seeds came from Malawi. The branches with inflorescences were placed in jars filled with tap water and maintained at room temperature (25 °C ± 5 °C). Flowers were produced by these branches for up to eight weeks, but our experiments were based on flowers produced in the first four weeks after the branches were placed in water. During this period a number of preliminary optimization and standardization tests (data not shown) were also undertaken.

2.3. Pollen viability tests

A number of staining methods were assessed to distinguish between fresh and dead pollen. Pollen was killed by spreading a small amount of the pollen mixture into a 100% ethanol droplet on a glass microscope slide, which was then carefully heated repeatedly (two or three times) with a flame (Sheffield et al., 2005). Five different staining techniques were used to test for pollen viability. (i) 1% aqueous solution of 2,3,5-triphenyl tetrazolium chloride (TTC) and (ii) 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 (Sheffield et al., 2005; Zeng-Yu et al., 2004). (iii) Aniline blue–lactophenol staining solution which 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). The pollen grain was considered viable if it turned blue (Kearns and Inouye, 1993; Khatun and Flowers, 1995; Zeng-Yu et al., 2004). (iv) Iodine and potassium iodide was prepared by dissolving 80 g potassium iodide KI and 10 g iodine I in 100 ml distilled water (Kearns and Inouye, 1993). Black-stained pollen was considered viable. (v) A fluorochromatic reaction (FCR) test, where fluorescein diacetate was dissolved in acetone (2 mg/ml) and used at 10^{-6} mol/l in 0.8 mol/l sucrose (Kearns and Inouye, 1993; Khatun and Flowers, 1995; Zeng-Yu et al., 2004).

Pollen was viewed using an Olympus AX70 Fluorescence Microscope. Viable and non-viable pollen grains were counted in each field of view for a total count of no less than 300 pollen grains. Non-viable pollen grains, which remain unstained, were distinguished from viable grains. Staining percentage was determined by dividing the number of stained pollen grains by the total number of pollen grains per field of view and expressed as a percentage after being normalized by angular transformation. 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. Fresh and dead pollen within each treatment were compared using the *t* test.

2.4. In vitro pollen germination

Bulk fresh pollen grains were collected one day 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) IAA [4 mg/l]; (3) basal medium comprised of 0.8 mmol/l sucrose + 0.7 mmol/l H_3BO_3 + 1.3 mmol/l $Ca(NO_3)_2.4H_2O$ + 1% agar, hereafter referred to as M, modified from Zeng-Yu et al. (2004).

Pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain (Tuinstra and Wedel, 2000). Two drops (2 µl each) consisting of a mixture of media and pollen were placed at different points on a glass slide and covered with cover slips. 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 a percentage and normalized by using 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.

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 female flower opening with pollen grains collected one day after anthesis. Self-pollinations were effected by brushing pollen from 2 to 4 anthers, taken from different flowers of the same inflorescence, on the stigma. Cross-pollinations were similarly effected using pollen from different plants. Hand-pollinated flowers were collected at 2, 4, 6, 8, 24, 48, 72 and 96 h after pollination (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 (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 measured using fluorescence microscopy. The differences in pollen tube length between self- and cross-pollinated flowers at two hourly intervals were compared using a *t* test. Bonferroni correction was applied by dividing the alpha value (0.05) by the number of comparisons (*n*).

3. Results

3.1. Pollen viability test

Tetrazolium salt (TTC) stained fresh pollen bright red and successfully ($P < 0.05$) distinguished between fresh and dead

Table 1
Percentage of stained fresh and dead pollen treated with 2,3,5-triphenyltetrazolium chloride (TTC), 2,5-diphenyl monotetrazolium bromide (MTT), iodine potassium iodide (IKI), the fluorochromatic reaction (FCR) or aniline blue. Data are means (± S.E) for five slides with two fields of view each (N=10).

Test	Fresh pollen	Dead pollen	t	P
TTC	95.4±1.1	22.3±3.8	3.20	0.001*
MTT	92.1±2.3	91.3±1.4	0.8	0.481
FDA	76.4±1.8	71.0±6.4	0.45	0.684
IKI	97.0±1.5	97.8±1.6	1.73	0.182
Aniline blue–lactophenol	93.9±1.2	79.0±2.7	10.7	0.002*

The mean percentages of stained fresh and dead pollen grains within each treatment were compared using t tests.

pollen (Table 1). Staining with 2,5-diphenyl monotetrazolium bromide (MTT), analine blue–lactophenol and IKI did not distinguish between the two types of pollen (Table 1). In the fluorochromatic test, both fresh and dead pollen showed fluorescence and the variation between individual grains was too great to allow fresh and dead pollen to be distinguished reliably (Table 1).

3.2. In vitro pollen germination and pollen tube growth

3.2.1. Pollen germination

Pollen from male flowers germinated significantly ($P<0.001$) better in medium M compared to the control and IAA (Fig. 2A). Media containing IAA did not increase pollen germination significantly over the control (Fig. 2A). Pollen from hermaphrodite flowers had a lower germination rate compared to pollen from

male flowers, without significant differences in germination among treatments (Fig. 2B).

3.2.2. Pollen tube growth

Pollen tubes from male flowers were significantly ($P<0.001$) longer in medium M than those in the IAA and control treatments (Fig. 2C). Pollen from hermaphrodite flowers had shorter pollen tubes than those from male flowers, without significant differences in pollen tube length among treatments (Fig. 2D).

3.2.3. In vivo pollen germination and pollen tube growth

In both the self- and cross-pollination treatments a large number of pollen grains germinated on the stigma (Fig. 3A, B, G, and H). The average length (± S.E) of *J. curcas* styles in the study sample was 1300±46.8 μm. Following self- and cross-pollination it took about 4 h for the pollen tube tips to reach the middle of the styles, 6 h to reach the base of the style, and they entered the ovary within about 8 h. Although the mean pollen tube length and growth rate were slightly higher in the cross-pollinated than in the self-pollinated flowers (Fig. 4A and B), these differences were not significant when the Bonferroni correction was applied to P values obtained from multiple t tests. The number of pollen tubes reaching the base of the style was often substantially less than the number in the stigma (Fig. 3C and I). The mean number of pollen tubes in the style was not significantly different between cross- and self-pollination treatments (Fig. 4C).
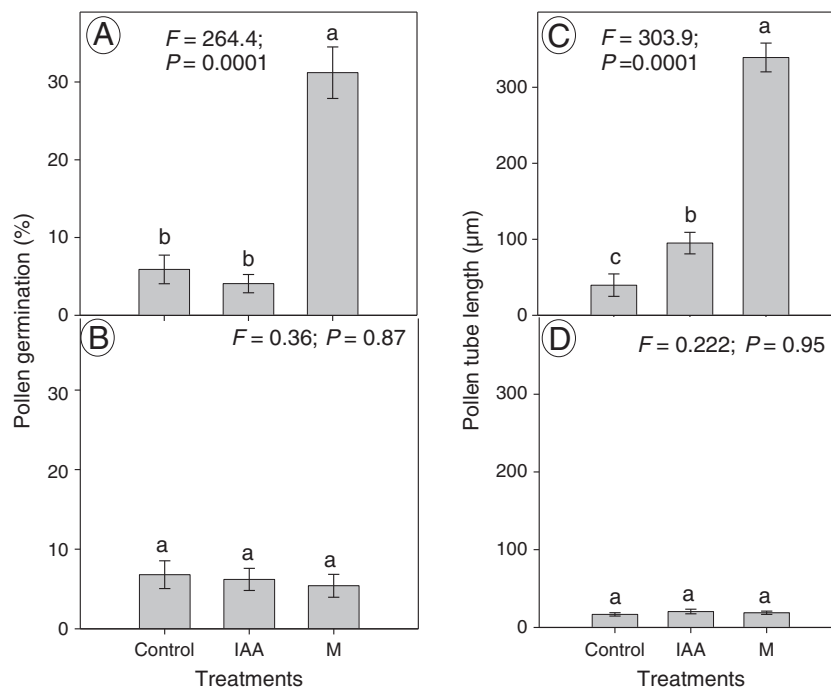

Fig. 2. Mean *in vitro* germination percentage and pollen tube growth of *Jatropha curcas* flowers. A and C: pollen from male flowers; B and D: pollen from hermaphrodite flowers. Standard error (±) bars with different letter(s) are significantly different from each other according to Tukey's test (P<0.05).
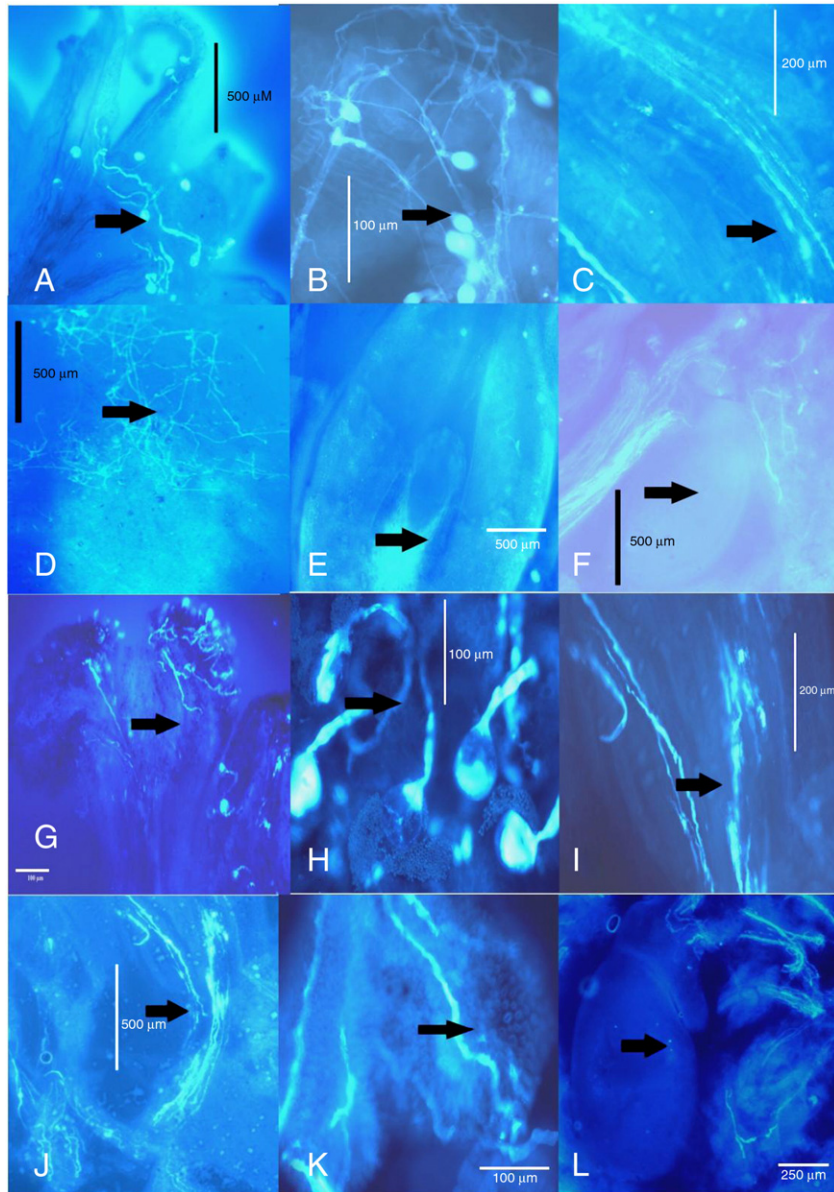
Fig. 3. *In vivo* pollen germination and pollen tube growth from (A–F) self-pollinated and (G–L) cross-pollinated *Jatropha curcas* flowers, visualized using fluorescence microscopy. In both A–E and G–K phases are developmental stages within 0–8 h after pollination (HAP). Phase A and G: pollen germination on the stigma, Phases B–C and H–I: a large number of pollen germinated on the stigma papillae and produced pollen tubes that grew into the style (arrows), Phase D and J: pollen tube had entered the ovary, Phase E and K: pollen tube had penetrated the ovule, Phase F and L: developing embryo 72 HAP. Scale bars are shown in each panel.

4. Discussion

Only tetrazolium salt (TTC) successfully differentiated between fresh and dead pollen in *J. curcas*. Thus, the staining percentage of the fresh pollen (95%) by TTC can be considered to represent the pollen viability percentage in the study sample. These findings concur with those of Change-Wei et al. (2007) who used TTC to evaluate pollen viability in this plant. It was concluded that pollen viability was high 9 h after anthesis and declined 33 h later. Kaur et al. (2011) reported 71% pollen viability in *J. curcas* using this technique, observing that viable pollen stained purplish pink and non-viable pollen remained unstained. Our experiments showed that fresh and dead pollen could not be differentiated by staining with iodine potassium

iodide (IKI) or fluorochromatic reaction (FCR). These results contradict the findings of Sasikala et al. (2009) who studied pollen fertility in ten *Jatropha* spp. and in an interspecific hybrid (*J. curcas × J. integerrima*) using potassium iodide (KI) dye. On the assumption that fully-stained pollen grains were fertile, they concluded that nine of these species had pollen fertility more than 84%, the hybrid 97%, and *J. tanjorensis* 0.16% which amounts to near sterility. In addition, Li et al. (2010) successfully tested pollen viability using the FCR technique. However, our results showed that staining with 2,5-diphenyl monotetrazolium bromide (MTT) and aniline blue–lactophenol did not differentiate between fresh and dead pollen and cannot be relied upon to determine pollen viability in this plant. A possible explanation for these results is that vital stain reaction may
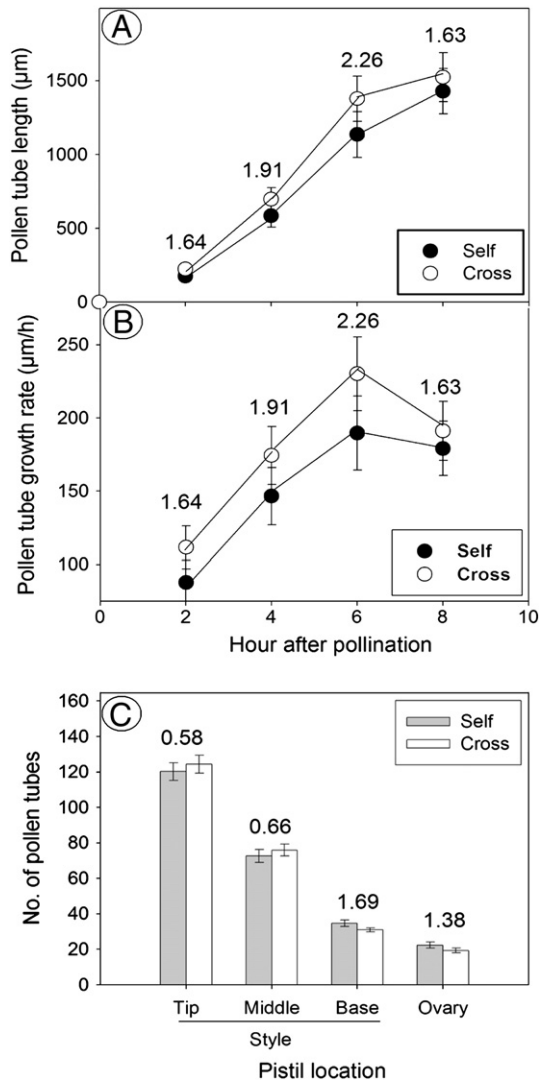
Fig. 4. *In vivo* pollen tube growth following self- and cross-pollination of *Jatropha curcas* flowers, (A) pollen tube length 2, 4, 6 and 8 h after pollination; (B) pollen tube growth rate 2, 4, 6 and 8 h after pollination; (C) number of pollen tubes in tip, middle and base of the style and ovary. A–C values are means±S.E (n = 10). Values above symbols and bars are *t* statistical values. None of these are significant after Bonferroni adjustment of the alpha value of 0.05.

(2009) to test pollen viability in *J. integrrima*. However, the objectives from these viability studies varied from comparing species or testing for natural hybridization within the genus *Jatropha*, or performing palynological studies on different plant species including species from the genus *Jatropha*. In this study, we tested pollen viability using different techniques applied to *J. curcas* pollen from the same source plants and, importantly used both fresh and dead pollen in the viability test to assure accuracy of the obtained results.

Pollen from male flowers of *J. curcas* germinated better in medium M, containing sucrose, calcium and boric acid as the main components, than in the control and IAA treatments. This agrees with Li et al. (2010) who reported a 71% pollen germination rate for *J. curcas* pollen in a medium containing sucrose, calcium and coconut water. In another study, Lyra et al. (2011) studied the *in vitro* pollen germination of *J. ribifolia* and *J. mollissima* in a gelled culture medium containing 1% agar and different sucrose concentrations. They reported rapid pollen germination and long pollen tube growth with 10% sucrose for both species. The germination index tended to decrease with increasing sucrose concentrations. Our observation showed that IAA did not increase pollen germination over the control. Furthermore, pollen tubes were longer in medium M than in the IAA-containing medium and were significantly longer in both treatments compared to the control treatment. This is partially in line with Wu et al. (2008a) who reported a distinct effect of exogenous IAA in *Torenia fournieri* L., which resulted in straighter and more slender pollen tubes compared with the control. However, pollen from hermaphrodite flowers had a lower germination rate and shorter pollen tube length compared to male flowers with no differences in germination between treatments. The possible explanation for these results could be attributed to male dysfunction as a result of indehiscent anthers or sterile pollen of the hermaphrodite flower. Male sterility has been reported in interspecific hybrids involving *Jatropha* species. Sahai et al. (2009) confirmed pollen sterility of hermaphrodite flowers in six accessions of hybrids resulting from crosses between *J. curcas* and *J. gossyfolia*. Liu et al. (2007) reported microspore abnormality either at the tetrad stage or in the early stage of microgametophyte development in *J. curcas*.

In *in vitro* experiments involving self- and cross-pollination treatments a large number of pollen grains germinated on the stigmatic exudates and formed callose, indicating good growth of pollen tubes. However, few of the pollen tubes were observed to elongate from the stigmatic exudates to the style. The average number of pollen tubes was only slightly higher in the cross- than in the self-pollination treatments. In addition, 6 h after pollination, the average pollen tube length and growth rate were slightly higher in the cross- compared to the self-pollinated flowers, but this was not significant after correction for multiple comparisons. Previous studies have suggested that self-pollen tubes can grow slower or have higher rates of attrition than cross-pollen tubes (Aizen et al., 1990; Cruzan, 1989; Weller and Ornduff, 1989; Horsley and Johnson, 2007; Li et al., 2008). Commonly the pattern of pollen tube growth is very similar in styles of self- and cross-pollinated flowers (Fenster and

vary from species to species. For example, negative results have been reported for various staining techniques on different plant species. In *Festuca arundinacea* (Poaceae), Zeng-Yu et al. (2004) were unable to distinguish between fresh and dead pollen using TTC, MTT, FCR and aniline blue; in some *Prunus* spp. (Rosaceae), heat-killed pollen was intensely stained by MTT (Parfitt and Ganeshan, 1989); and in *Leymus chinensis* (Poaceae), TTC did not stain dead pollen but IKI stained the dead pollen in a similar manner to fresh pollen (Huang et al., 2004). Pollen viability in *J. curcas* and nine other *Jatropha* spp. was apparently successfully tested by Basha and Sujatha (2009) using a mixture of equal amounts of aceto-carmine and glycerol. Lyra et al. (2011) successfully used aceto-carmine, acetic orcein and cotton-blue stains to test pollen viability in *J. ribifolia* and *J. mollissima*, while Noor et al. (2004) used techniques similar to those used by Basha and Sujatha

Sork, 1988; Hessing, 1986; Ockendon and Gates, 1975; Sarr et al., 1983). Recently, *in vivo* studies on *Jatropha* species were reported by Parthiban et al. (2009) who performed a hybridization programme between *J. curcas* as female parent and the wild species *J. integrrima*, *J. podagrica*, *J. villosa*, *J. tanjorensis*, *J. gossypifolia*, *J. glandulifera*, *J. multifida* and *J. maheshwarii* as pollen donors. Self- and cross-pollinations were made and the pollen tube growth was recorded using fluorescent microscopy after staining with aniline blue. In self-pollinated intraspecific crosses involving *J. curcas*, the pollen germination rate was 82%; pollen tubes were produced; entered into the stigma, style and eventually into the ovary 1 h after pollination. It was reported that 43 pollen grains out of 56 produced pollen tubes and only six of them entered into the ovary. Similar results were obtained by Kumar et al. (2009) while investigating pollen-pistil interaction between cultivated *J. curcas* and some wild species (*J. gossypifolia*, *J. podagrica* and *J. villosa*). In their study fertilization with self-pollination in *J. curcas* was completed within 1 h after pollination, no crossability barriers were noticed, seed set was 88% and the progeny exhibited 85% germination in sand media. Rates of pollen tube growth in these studies were thus significantly faster than those recorded in the present study in which pollen tubes took about 8 h to reach the ovary (Fig. 4B).

Pollen viability and the efficiency of pollen transfer partially determine the reproductive success of a species. *In vitro* germination of pollen grains is necessary for various biotechnological manipulations. Studies on pollen viability of *J. curcas* are crucial for conservation, interspecific hybridization and management of cultivars. Such studies can be used to overcome crossing barriers in interspecific hybridization in the genus *Jatropha*. This leads to the possibility of transferring the genes controlling useful traits such as femaleness and oil content. In conclusion, out of the five vital stains used, TTC proved to be effective in determining pollen viability in this plant. Both types of pollination maintained fertility as measured by penetration of an ovule by a pollen tube, and are thus in agreement with our previous findings that fruits arising from self-pollination are almost as numerous and large as those arising from cross-pollination (Abdelgadir et al., 2009). It is unclear exactly which factors determine the male dysfunction in hermaphrodite flowers of *J. curcas* and further investigations are required to shed light on the function of this type of flower.

Acknowledgements

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Edited by JC Manning